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# Gas Vesicles

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## INTRODUCTION

Of the many types of organelles and subcellular structures that have evolved in procaryotic organisms, only one, the gas vesicle, contains a gas-filled space. Gas vesicles occur almost exclusively in microorganisms from aquatic habitats, in which, by lowering the density of the cells, they provide buoyancy. They have been found in over 150 species of procaryotes (241, 242), from at least 5 of the 11 phyla of bacteria and 2 of the phyla of archaea described by Woese (276). In all of the organisms investigated, gas vesicles have a similar morphology and are constructed from a homologous protein. They show considerable diversity, however, in one aspect, their width. The understanding of this has required studies of the chemistry and mechanics of gas vesicles, the molecular biology of the proteins that form them, their buoyancy-providing role, and the ecology

of the organisms in which they occur. The purpose of this review is to describe these studies and explain how natural selection has shaped the gas vesicle in various procaryotes.

In concentrating on the study of a small corner of biology, there is always the hope that one may uncover information that can be applied more widely in the subject. The studies on gas vesicles have made contributions to a number of fields in microbiology: they have explained how certain aquatic microorganisms form surface waterblooms or position themselves in lakes (127, 231); they have provided a method of measuring cell turgor pressure in bacteria (178, 230, 239); and they have provided a means of determining the rate at which gases diffuse into cells (246).

The studies on natural selection of the gas vesicle, rather than establishing new principles, have drawn heavily on existing ones from many other areas of science. In doing so, they have provided an account of each step in the process by which selection for a specific trait in the phenotype may enrich the incidence of the genotype that specifies it. This approach might be used to analyze the evolution of other structures.

### Gas Vacuole and Gas Vesicle

Gas vesicles are the components of gas vacuoles, which were discovered in cells of waterbloom-forming cyanobacteria by German microbiologists nearly a century ago (3, 113, 208). Klebahn (113) showed that gas vacuoles contain gas and provide cells with buoyancy (114–116). He found that they could be distinguished from other cell constituents in the light microscope by their disappearance under pressure (114). Structures with similar properties in other planktonic bacteria were demonstrated by Lauterborn (146). These early studies on gas vacuoles were reviewed in 1941 by Fogg (62).

The current era of research on gas vacuoles was launched in 1965, when Bowen and Jensen (20) found that the gas vacuoles in cyanobacteria were made up of stacks of cylindrical vesicles measuring 75 nm in diameter and up to 1.0 μm in length. The cylinders, closed by conical ends, were formed by a single wall layer only 2 nm thick. Cells that had been subjected to a pressure of 0.7 MPa (7 bars) had lost these cylindrical structures; in their place were paired membranes, which were correctly interpreted as the collapsed vesicles. Bowen and Jensen called the structures gas vesicles. Over the next 5 or 6 years the principal properties and functions of gas vesicles were determined, and I reviewed these findings for this journal in 1972 (231). Many of them were based on flimsy evidence, but, as this review will show, almost all of them remain tenable. The present state of knowledge can be summarized as follows.

Gas vesicles are inert, hollow, gas-filled structures, formed solely from protein (104, 256). The main constituent is a small hydrophobic protein arranged in a linear crystalline array (15) along ribs that form the cylindrical shell and its conical caps (108). A second protein, which has a repeating amino acid sequence, adheres to the outside of the ribs and stabilizes the structure (258). The genes that encode these proteins have been isolated and sequenced, first in cyanobacteria (37, 210) and then in halobacteria (39, 90). The gas vesicle is freely permeable to gases and cannot be inflated by gas (228). The gas space must be formed by the way in which the proteins assemble; it rapidly fills with gas by diffusion. Liquid water is kept out by surface tension at the hydrophobic inner surface. Pressures on the gas vesicle are borne by the rigid wall and do not compress the gas inside the structure (244). The gas vesicle is capable of withstanding pressures of several bars, but at a certain critical pressure it collapses irreversibly (230). The critical pressure is determined by the mechanical properties of the protein (252) and by the diameter of the cylindrical structure (86, 254).

Gas vesicles occur almost exclusively in procaryotes from aquatic habitats. Their function is to provide buoyancy (231), which allows aerophilic bacteria to float into oxygenated surface waters (171, 234) and enables cyanobacteria to float up toward the light; some cyanobacteria and photosynthetic bacteria regulate buoyancy provided by their gas vacuoles and they stratify in layers below the water surface. The mechanisms of buoyancy regulation in cyanobacteria may involve modulation of gas vesicle gene expression and the destruction of gas vesicles by turgor pressure (which can be measured by using gas vesicles as pressure probes); they also involve counteracting effects of changes in carbohydrates and other dense substances. These mechanisms are affected by light, and this explains how these organisms regulate their vertical distribution in natural waters (248).

It is in the natural environment of ponds, lakes, and seas that gas vesicles have evolved; it is proposed that two counteracting factors have been involved in the natural selection of their width. The amount of gas space enclosed by gas vesicle protein increases with the width of a gas vesicle. There should therefore have been selection for wide gas vesicles, which provide buoyancy with greater economy than narrow ones. For mechanical reasons, however, the critical collapse pressure of a gas vesicle varies inversely with its width. The highest pressure to which organisms will be exposed in their natural habitat must therefore have set an upper limit on the width of their gas vesicles. Put simply, the greater the depth of the water column in a lake or sea (and the higher the cell turgor pressure), the narrower the gas vesicle must be to withstand the pressure.

Natural selection, the differential survival and perpetuation of phenotypes, requires inheritance through genotypes that encode the phenotype in the next generation (55). The width of the gas vesicle is genetically controlled; it is possibly determined by the sequence of the main gas vesicle protein, but it may be modulated by the products of some of the other genes involved in gas vesicle production. The results of transformation experiments in halobacteria (58, 91) strongly suggest that a cluster of only 14 genes carries all the information required for the production and regulation of gas vesicles. The natural selection of the gas vesicle may therefore be limited to the few phenotypic characters encoded by these genes. The story of the natural selection of gas vesicles is no longer open ended but circumscribed; it is now necessary to consider the details. The account that follows deals mainly with gas vesicles of cyanobacteria. Much of what has been learnt about their structure and properties must be applicable to gas vesicles in other procaryotes, but the way these organelles interact in the growth and ecology of other organisms is largely unexplored.

## MOLECULAR STUDIES ON GAS VESICLE PROTEINS

There are at least two types of protein present in the cyanobacterial gas vesicle; a small hydrophobic protein (GvpA) forms the ribs of the main structure, and a larger, more hydrophilic protein (GvpC) stabilizes the structure. There may, in addition, be variants of the small protein present. Genetic analysis of halobacteria gives evidence of the same two classes of protein and indicates that there are as many as 14 genes involved in gas vesicle formation. The products of some of these genes may form minor components of the gas vesicle while others may be involved in assembly or regulation. A transformation system that provides the means of investigating the role of these genes has recently been developed.

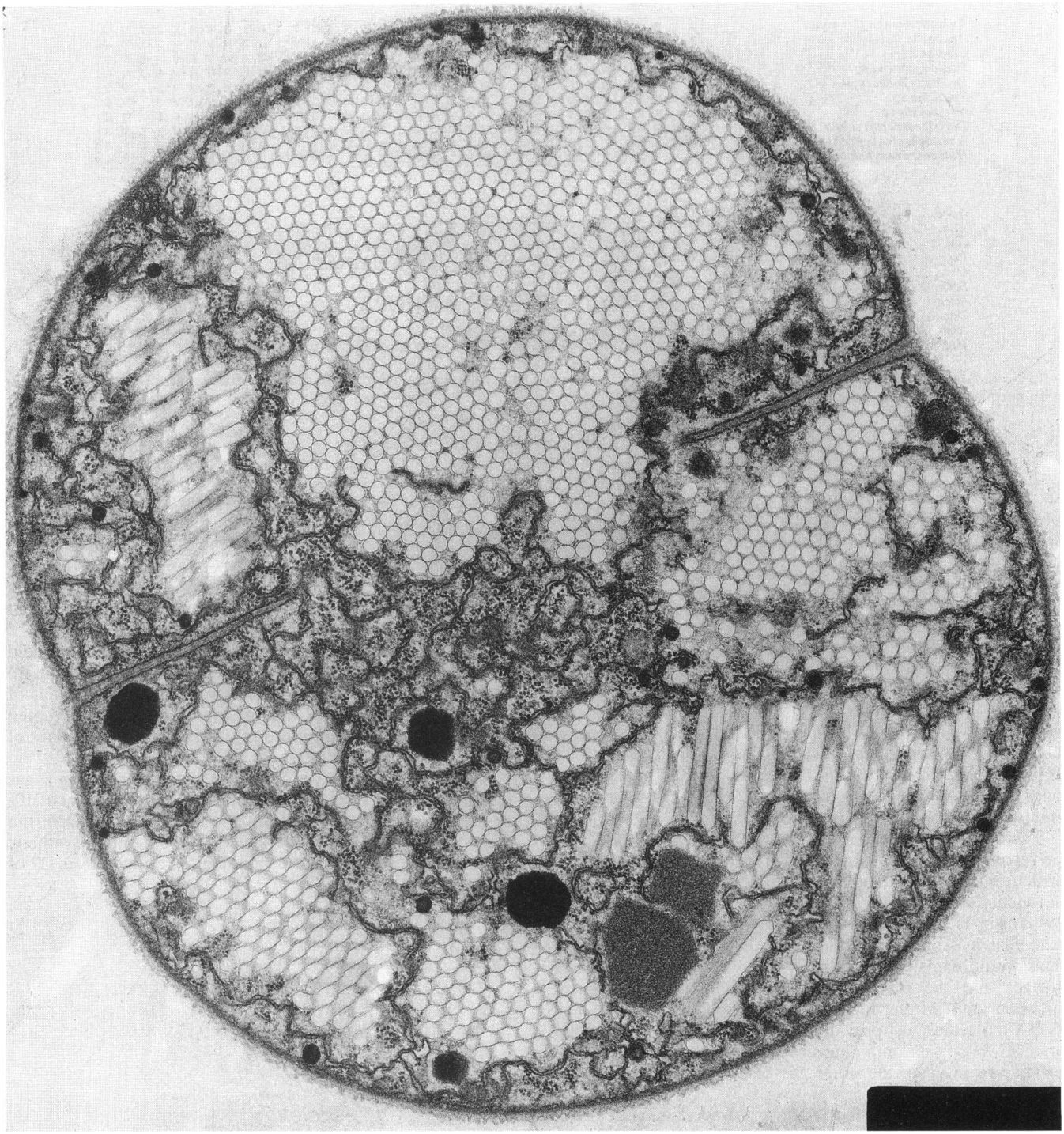

FIG. 1. Transverse section of a dividing cell of the cyanobacterium *Microcystis* sp. showing hexagonal stacking of the cylindrical gas vesicles. (Micrograph by H. S. Pankratz.) Magnification, ×31,500.

## Gas Vesicle Proteins in Cyanobacteria

**The small rib protein, GvpA.** All cyanobacterial gas vesicles so far analyzed contain a protein (GvpA) of about 7.4 kDa that forms the main mass of the structure and must be responsible for many of its properties. The complete amino acid sequence of this protein has been determined by performing automated N-terminal analysis on samples of gas vesicles and peptides purified from tryptic digests of gas vesicles. The first sequences, from *Microcystis aeruginosa* gas vesicles (270), revealed the first four residues, Ala-Val-Glu-Lys, and short sequences of several other nonoverlapping peptides. With improvements in automated sequencing, we determined the first 64 residues from three overlapped peptides from the gas vesicles of *Anabaena flos-aquae* (227) and then the final overlap with the 15-residue C-terminal peptide, indicating a 70-residue protein (87, 226). With gas phase sequencing it became possible to obtain

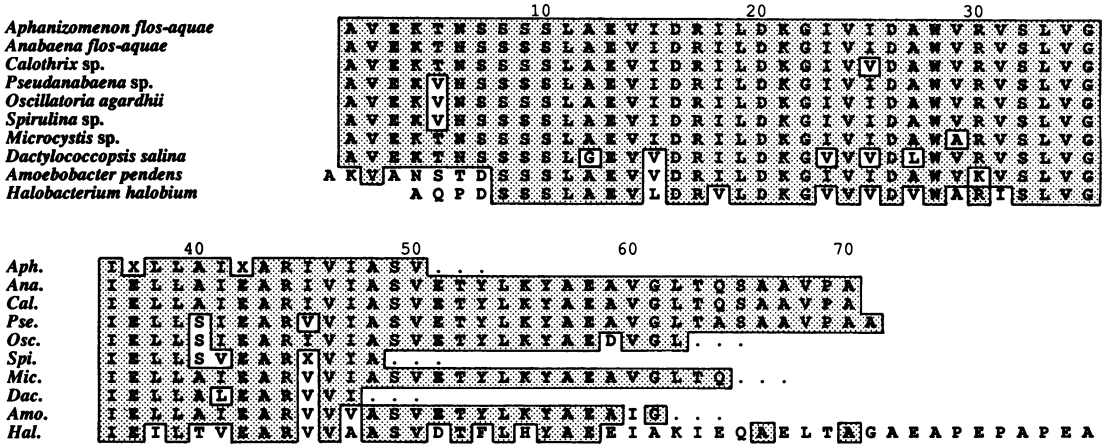


FIG. 2. Amino acid sequences of GvpA from cyanobacteria, a purple bacterium, and a halophilic archaeon. Modified from Griffiths et al. (78) with permission from the publisher.

sequences of over 60 residues in a single run directly from preparations of purified gas vesicles (87). In this way extensive sequences were obtained for this protein from eight species of cyanobacteria (78, 87), two species of halobacteria (209, 226), and a purple bacterium (78) (Fig. 2).

The molecular mass of this protein in *Anabaena flos-aquae*, 7397 Da (84), corresponds to that of the crystallographic unit cell repeating along the ribs of the structure, about 7.5 kDa (15). The GvpA is left in the ribbed structure that remains when the only other detectable protein component of the gas vesicle (GvpC) is removed by rinsing with solutions of detergent (258) or 6 M urea (83). It is therefore concluded that GvpA forms the ribs from which the gas vesicle is constructed. It has been calculated that, if these two proteins are the only ones present, GvpA accounts for about 90% of the mass of the *Anabaena* gas vesicle (see below).

The amino acid sequence of the GvpA molecule has yet to be reconciled in detail with its low-resolution crystallographic structure (see, The Subunit, below); conflicting predictions of secondary structure are made by the various programs of the Wisconsin Package (45). Hydropathy plots (see below) indicate that it is one of the most hydrophobic proteins known. This would explain the hydrophobic properties of the inner surface and the possible hydrophobic interactions formed between and within GvpA molecules. The 70-amino-acid GvpA molecule must provide the essential features of the gas vesicle, whose gas-containing structure, shape, and basic properties remain when the other gas vesicle protein is removed (258).

In normal sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), most of the GvpA remains in the loading well (84, 258), although a small portion of it may move to the top of the separating gel, where it forms a family of "polymers" of the 7.4-kDa polypeptide (Fig. 3). They have been identified as GvpA by Western immunoblotting (259) and by N-terminal sequencing (78). These polymers must be undissociated fragments of the gas vesicles, perhaps linear arrays of GvpA from the ribs. *Anabaena* gas vesicles do dissolve to form a clear solution in 80% formic acid (227), but the protein precipitates on subsequent dialysis and cannot subsequently be separated. Damerval et al. (35) reported that similar treatment of gas vesicles from *Pseudanabaena* sp. yielded a 7.5-kDa band by SDS-PAGE. Englert et al. (60) commented that an 8-kDa protein that reacts with antibodies to GvpA can be separated by SDS-PAGE from *Halobacterium* cells that have not yet assembled the protein but not from the gas vesicles themselves. It is possible, therefore, that the GvpA is modified in some way after assembly. Proteins from isolated gas vesicles have been separated by other electrophoretic systems (104, 231, 235), but their identity is uncertain.

It is important to resolve the nature of the bonds that hold the GvpA molecules together so tenaciously. If they are covalent linkages, they must occur close to the C terminus of the protein, because it is possible to obtain an uninterrupted sequence from the N terminus to within at least 6 residues of the C-terminal residue of GvpA (87).

**The encoding gene, *gvpA*.** The first gene encoding a gas vesicle protein was isolated by Tandeau de Marsac et al. (210) by probing a library of the genomic DNA isolated from the cyanobacterium *Calothrix* sp. strain PCC 7601 with a synthetic 29-mer oligonucleotide corresponding to residues 18 to 27 of

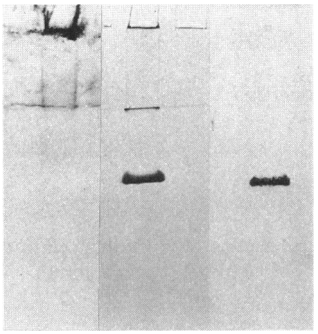

FIG. 3. Separation of the gas vesicle proteins, GvpA and GvpC, from *Anabaena flos-aquae* by PAGE, and identification by immunoblotting. Center lane: Coomassie blue-stained gel showing GvpA in the loading well and at the top of the separating gel, and the mobile band of GvpC in the separating gel. Left lane: blot probed with antibody to the N-terminal peptide of GvpA (A-V-E-K-T-N-S-S-S-S-L-A-E). Right lane: blot probed with antibody to a peptide that occurs three times in GvpC (A-Q-A-E-K-Q-A-Q-E-L). Reproduced from Walsby and Hayes (259) with permission from the publisher.

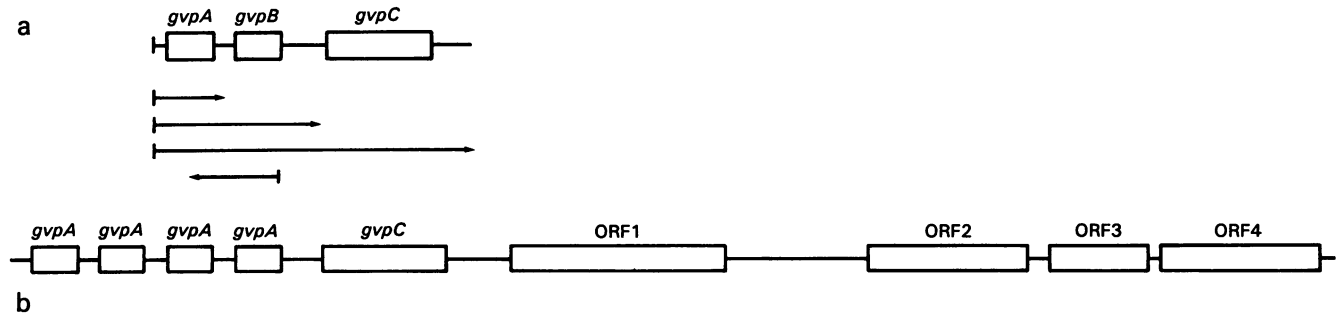


FIG. 4. Arrangement of *gvp* genes in cyanobacteria. (a) The *gvpABC* operon of *Calothrix* sp. (37), showing the transcripts (arrows) from the coding strand and an antisense transcript from the noncoding strand *gvpB* is renamed *gvpA2*. Redrawn from Csiszàr et al. (33) with permission from the publisher. (b) The cluster of *gvp* genes in *Anabaena flos-aquae*, which contains an operon of at least five *gvpA* genes (four are shown) and a single *gvpC* gene that is followed by a cluster of four ORFs that show homology to the following *gvp* genes in *Halobacterium halobium*: ORF1 (*gvpN*), ORF2 (*gvpJ* and *gvpA*), ORF3 (*gvpK* and *gvpJ*), ORF4 (*gvpF* and *gvpL*). Drawn to the same scale as panel a; the length shown is 6.25 kbp. Unpublished data of Kinsman and Hayes (109a).

the *Anabaena* amino acid sequence (Fig. 2). The gene, *gvpA*, encoded a 70-residue protein that showed very high homology to the *Anabaena* gas vesicle protein (Fig. 2). By using the *Calothrix gvpA* gene as a probe, the homologous genes have now been isolated and sequenced from two other cyanobacteria, *Anabaena* (259) and *Pseudanabaena* (35) species, a colorless bacterium, *Ancylobacter aquaticus* (21a), and two *Halobacterium* species (39, 90, 209).

In *Calothrix* species there are two genes in tandem repeat, originally termed *gvpA* and *gvpB* (37) but now redesignated *gvpA1* and *gvpA2*, respectively, because they encode identical 70-residue GvpA proteins; i.e., there is no distinguishable GvpB protein product (Fig. 4).

In *Pseudanabaena* sp., a filamentous cyanobacterium that produces rather small amounts of gas vesicles next to the cell septa, only one copy of *gvpA* has been found (35). In *Anabaena flos-aquae*, a planktonic cyanobacterium that constitutively produces large numbers of gas vesicles, there is evidence of at least five, and probably seven, copies of the *gvpA* gene in tandem repeat (82a), but all four of those so far sequenced encode identical 70-residue proteins (179a).

**The large outer surface protein, GvpC.** By sequencing downstream from *gvpA1* and *gvpA2* in *Calothrix* sp., a third open reading frame (ORF), *gvpC*, was found by Damerval et al. (37). The deduced amino acid sequence indicated a protein of 162 amino acid residues; its function was unknown. A gene encoding a homologous but somewhat larger protein of 193 residues was found downstream of *gvpA* in *Anabaena flos-aquae* (84) (Fig. 5).

SDS-PAGE of purified *Anabaena* gas vesicles yielded only a single mobile band indicating a protein of about 22 kDa. Because only a single amino acid sequence, that of GvpA, had previously been obtained from intact gas vesicles, this band was initially confused with GvpA (227), but N-terminal amino acid analysis of the eluted protein showed a sequence identical to the deduced N-terminal sequence of the *Anabaena gvpC* gene product (84). Sequence analysis of seven peptides obtained by trypsin digestion of the protein present in isolated gas vesicles confirmed that the product of this gene was a component of the gas vesicle. The size of the separated protein also corresponded to the molecular mass calculated from the inferred sequence of the 193-amino-acid gene product, 21,985 Da (Fig. 5).

These GvpC proteins are quite different from GvpA. They are rich in glutamine, alanine, and glutamic acid, which together account for over 40% of the residues, and they contain over 6% phenylalanine, which is absent from GvpA. Overall they contain a preponderance of hydrophilic residues; the contrasting natures of the two proteins are illustrated by the hydropathy plots (Fig. 6). The most striking feature of GvpC is the presence of a highly conserved motif of 33 amino acid residues, which forms tandem repeats four times in the *Calothrix* GvpC and five times in the *Anabaena* protein. As

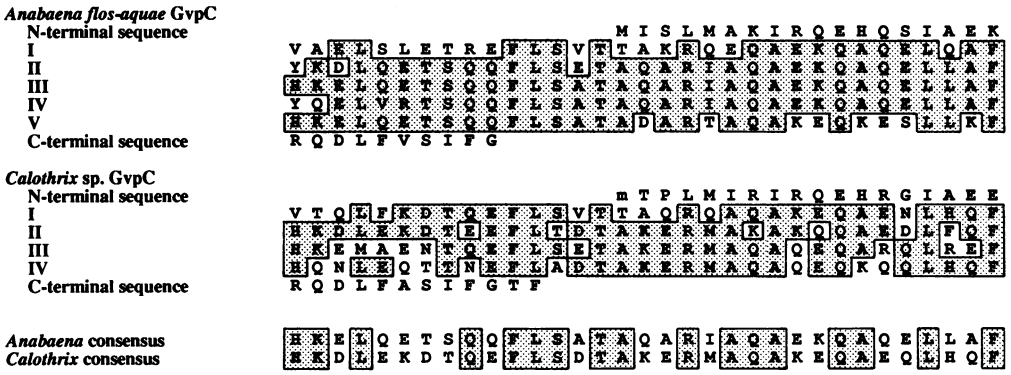


FIG. 5. Amino acid sequences of GvpC from *Anabaena flos-aquae* (84) and *Calothrix* sp. (37), showing the 33-amino-acid repeats. Identical residues are boxed; the consensus sequences are shaded and compared below. Modified from Walsby and Hayes (259) with permission from the publisher.

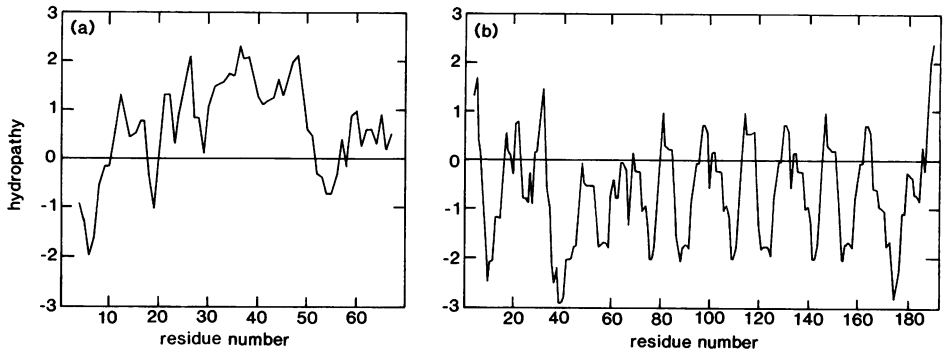


FIG. 6. Hydropathy plots of GvpA and GvpC from *Anabaena flos-aquae*. Modified from Walsby and Hayes (259) with permission from the publisher.

discussed below (Speculations on How GvpC Binds to Gas Vesicle Ribs), they are believed to make a periodic interaction with the crystalline structure of the ribs formed by GvpA (258). Flanking the tandem repeats are sequences of 18 residues at the N-terminal end and 10 (in *Anabaena flos-aquae*) or 12 (in *Calothrix* sp.) residues at the C-terminal end. All three sections of the molecule show homologies between the two species (Fig. 5).

**Function of GvpC: strengthening the gas vesicle.** We found that GvpC could be removed from intact gas vesicles isolated from *Anabaena flos-aquae*; this was done by rinsing them with a 2% solution of the detergent SDS. What remained were the gas-filled shells (formed by the ribs of GvpA), which floated and could be separated from the detergent solution by centrifugation. They resembled entire gas vesicles in most of their general properties, but they were weaker and collapsed at a much lower critical pressure (258). The removal of GvpC indicated that this protein must be located on the outside of the structure. The weakening of the structure could, in theory, have been caused either by the effect of the detergent on the remaining GvpA or by the removal of GvpC (258).

The outer protein could also be removed from isolated gas vesicles with 6 M urea (83), which resulted in similar weakening. GvpC could then be assembled back onto the stripped gas vesicle shells by dialyzing the urea away. Hayes et al. (83) synthesized *Anabaena* GvpC by recombinant technology: the protein was formed in inclusion bodies inside the cells of *Escherichia coli* containing the *Anabaena gvpC* gene on an expression vector. When this purified recombinant protein was assembled onto stripped gas vesicles containing only GvpA, the critical pressure of the gas vesicles increased from 0.19 to 0.56 MPa, within 3% of its original value (see Critical Pressure below) (see Fig. 20). The weakening and recovery of strength were reversible and depended on the amount of GvpC that was bound (83, 258). These experiments proved that the function of GvpC is to increase the strength of the gas vesicle structure.

Kinsman and Hayes (109a) have now used recombinant techniques to produce a series of three modified GvpCs containing the first 2, 3, and 4 of the 5 33-residue repeats between the 18 N-terminal residues and 10 C-terminal residues of the *Anabaena* protein (Fig. 5). These proteins will, like the complete GvpC, bind to and restrengthen stripped gas vesicles. We are now investigating how the number of repeats affects the efficiency of the protein.

The GvpC must be indispensable to the survival of gas vesicles in *Anabaena* sp. because without it all of the gas vesicles would be collapsed by the 0.4-MPa (4-bar) turgor pressure in the cells, even without the additional hydrostatic pressure of the water column in a lake. As discussed below (see Buckling Pressure), it might be feasible to make gas vesicles of the required strength without GvpC, but they would have to be much narrower and would therefore be less efficient in providing buoyancy.

The weakening caused by removal of GvpC has also been demonstrated with the gas vesicles of *Microcystis* sp. (258) and *Aphanizomenon* sp. They will bind recombinant *Anabaena* GvpC, and this partially restores their strength (22).

**GvpC in other cyanobacteria.** In a survey of 26 strains from six genera of gas-vacuolate cyanobacteria, Damerval and his coworkers (34) detected genes that hybridized to the *Calothrix gvpA* gene in all of them; however, only 12 strains contained genes hybridizing to the *Calothrix gvpC* gene. Those strains that did were said to make gas vesicles more abundantly. The strains that did not must either lack *gvpC* or possess a homolog (or functional analog) of the *gvpC* gene whose homology is too low to permit hybridization (34).

We have shown by direct electrophoretic analysis that the gas vesicles isolated from two unicellular cyanobacteria, two filamentous forms, and three heterocystous forms contain at least one additional protein (78). Amino acid sequence analysis showed that these proteins have a much lower homology to one another than do the GvpAs from the same organisms (compare Fig. 2 and 7). In one cyanobacterium investigated, *Pseudanabaena* sp., no GvpC has been detected on the isolated gas vesicles and no gene homologous to *gvpC* has been found, either by hybridization studies or by sequencing downstream from *gvpA* (35). It is conceivable that GvpC had been present but was lost during gas vesicle isolation and that the encoding gene is located further away from the *gvpA*. If these gas vesicles have no strengthening GvpC, however, it would be interesting to know how their critical pressure compares with that of other gas vesicles with similar width (254).

**Other *gvp* genes.** Elsewhere in the *Calothrix* chromosome is a gene that encodes a 71-residue protein that differs in only 4 residues from GvpA (33a). The amino acid sequence obtained by gas phase sequencing of entire *Calothrix* gas vesicles is identical to the sequence inferred from *Calothrix gvpA* (87), without any evidence of the four residues specified by the other *gvpA*-like gene.

Kinsman and Hayes (109a) have sequenced the 6.5-kb region downstream of *gvpC* in *Anabaena flos-aquae*; the sequence contains eight ORFs (ORF1 to ORF8), the first four of which show homology to *gvp* genes in *Halobacterium halobium*: the ORF1 product shows 40% identity to *Halobacterium* GvpN, the ORF2 product shares homology with GvpJ and GvpA, the ORF3 product shares homology with GvpK and

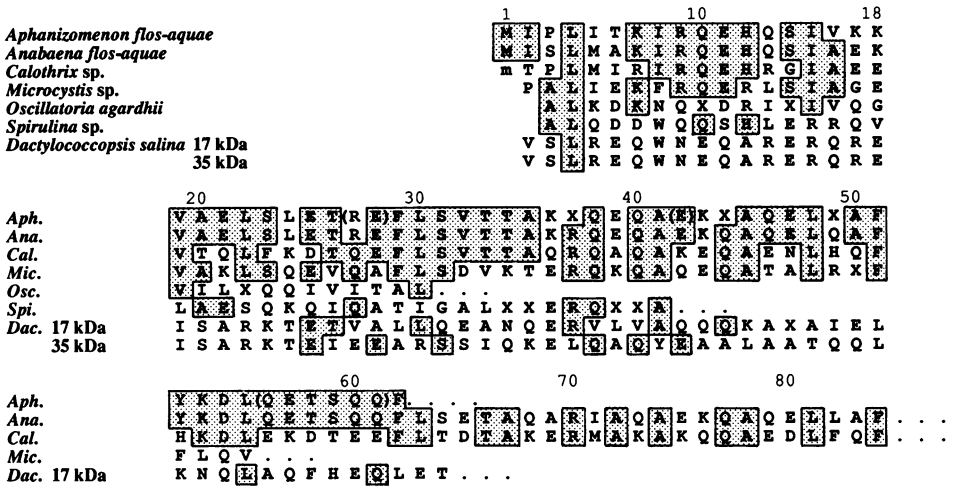


FIG. 7. Comparison of the N-terminal amino acid sequences of GvpC in seven cyanobacteria. Modified from Griffiths et al. (78) with permission from the publisher.

GvpJ, and the ORF4 product share homology with GvpF and GvpL. These products may therefore be involved in gas vesicle formation, as discussed in relation to *Halobacterium* gas vesicles (see Table 1). The next 4 ORFs show no homology to *gvp* genes.

**Relative proportions of gas vesicle proteins.** In order to determine the molecular structure of the gas vesicle, it is necessary first to identify all of the constituents and then to determine the proportions in which they occur. Proteins are the only constituents definitely identified in cyanobacterial gas vesicles. Analyses have been made for carbohydrates and lipids, but neither have been detected (103, 256). The phosphate content of isolated gas vesicles, analyzed by a sensitive $^{32}$P-labeling technique (258), is negligible (<0.002 mol/mol of GvpA).

Although GvpA and GvpC are the only proteins that have been detected in gas vesicle preparations, it is possible that others are present. The strongest candidates must be the products of *gvpJ* (and also *gvpM* in Halobacterium spp.), whose inferred amino acid sequences show some homology with GvpA. These and other proteins might, like GvpA, remain in the loading wells of the electrophoresis gel and thereby escape detection. There may also be SDS-soluble proteins that occur in such small amounts that they have not been detected by electrophoresis, though they cannot be present in more than trace amounts because even in heavily loaded gels the only mobile band found is GvpC (Fig. 3), and that represents only about 10% of the protein mass present.

It might be thought that evidence of other proteins could be obtained by analyzing the N-terminal amino acid sequences of proteins present in preparations of isolated gas vesicles; only a single sequence, that of GvpA, can be detected (227). With methods currently available it is possible to detect the sequences of two proteins simultaneously but only when they are present in roughly similar molar concentrations; the N-terminal sequence of GvpC, present at only 4 mol% GvpA, is undetectable (84). N-terminal sequencing is not very helpful in determining the amounts of major constituents either. The amount of the N-terminal residue (alanine) in the N-terminal peptide (Ala-Val-Glu-Lys) released from GvpA by proteolytic digestion of gas vesicles was equivalent to only 38% of the total calculated to be present (from data in reference 87, allowing for alanine content of GvpC). Although this represents a good yield of the peptide, it is not high enough to eliminate the possibility that substantial amounts of other proteins were present.

An approach that might be used to detect the presence of other proteins that cannot be separated from GvpA is to digest the purified gas vesicles with proteolytic enzymes, separate the peptide fragments by high-pressure liquid chromatography, and sequence them. Of the 16 peptide fragments we have analyzed, 9 had sequences attributable to GvpA (87, 227) and the other 7 were attributable to GvpC (84); there were other minor fragments that were not analyzed, however.

In summary, although there is no direct evidence of proteins other than GvpA and GvpC, we cannot rigorously exclude the possibility of other constituents. This should be remembered in interpreting the following information on the ratio of GvpA and GvpC.

In the *Anabaena* gas vesicle there are two amino acids—proline and tryptophan—that occur in GvpA but are absent from GvpC; three others—histidine, methionine, and phenylalanine—occur only in GvpC. These amino acids that occur exclusively in one protein or the other provide fortuitous specific labels, which can be used to determine the relative proportions of the two proteins in the gas vesicle.

It was shown that GvpC accounts for 8.9% of the gas vesicle mass by measuring the methionine content by a $^{35}$S-labeling technique and calculating the gas vesicle mass from the contained gas volume (258). In this method no assumptions are made about the identity of the remaining protein. In other determinations based on the relative abundance of phenylalanine or proline to other amino acids (84), it was calculated that if all of the remaining protein were GvpA, the molar ratio of GvpA to GvpC would be about 33:1. From the relative molecular masses of these two proteins, 7,397 and 21,985, GvpC would constitute 8.3% of the mass. We have recently demonstrated that some of the GvpC can be lost during gas vesicle isolation and that, if precautions are taken to prevent this, a molar ratio of 25:1 is obtained, in which case GvpC would account for 10.6% of the mass (22). This ratio supports a molecular model of the interaction between the two proteins (see Speculations on how GvpC Binds to Gas Vesicle Ribs, below, and Fig. 12).

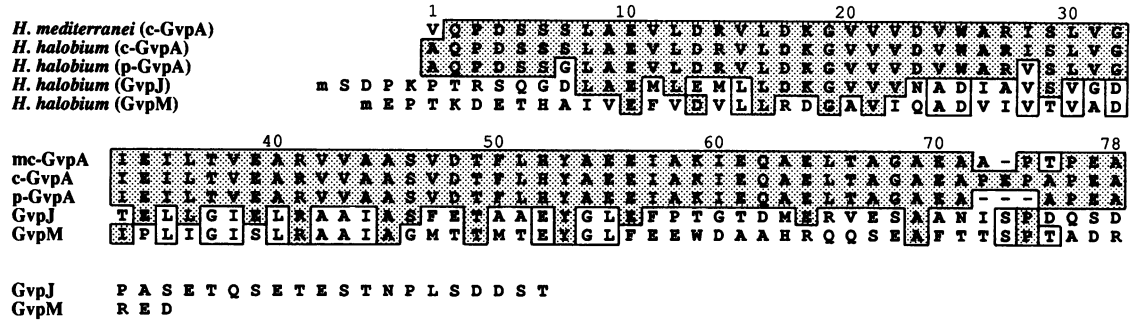


FIG. 8. Amino acid sequences of GvpA and other related gene products (GvpJ and GvpM) in halobacteria. Residues identical to those in c-GvpA are boxed and shaded; other identical residues are boxed but unshaded. (Data from Englert et al. [57] and Jones et al. [106].)

### Gas Vesicle Proteins in Other Bacteria

Amino acid sequencing of gas vesicles isolated from a purple bacterium, *Amoebobacter pendens* (78), shows the presence of a GvpA with a sequence that, although it differs in 6 of the N-terminal 8 residues, is otherwise highly homologous with GvpA in cyanobacteria (Fig. 2). There is a second protein in the isolated gas vesicles that can be separated by electrophoresis but shows no homology to cyanobacterial GvpCs (21a). The colorless bacterium *Ancylobacter aquaticus* contains a *gvpA* gene that encodes a 69-residue GvpA, which has a similar degree of homology to the cyanobacterial GvpAs: only 14 of the residues differ from the cyanobacterial consensus sequence, and of these 13 occur in other GvpAs. Amino acid sequencing of entire gas vesicles confirms that this is the major protein present (21a). Again, a second protein is present, with an electrophoretic mobility suggesting an $M_r$ of about 50,000 (132). Its N-terminal sequence also lacks sequence homology to other GvpCs; it is encoded by a gene located downstream from *gvpA* (21a). These results indicate great similarities between the GvpA proteins of gas vesicles in proteobacteria and cyanobacteria and a similarity in the possession but not the sequence of an additional protein. The phylogenetic implications of this are discussed below.

### Gas Vesicle Proteins in Halobacteria

Halobacteria have gas vesicles that are clearly homologous to those in cyanobacteria, but initial studies revealed some curious anomalies in their composition, formation, and stability which have now been resolved by sequence analysis of the gas vesicle proteins and the encoding genes. Gas vacuoles were discovered in halobacteria by Petter (171), and the constituent gas vesicles were first isolated from *Halobacterium salinarium* by Larsen et al. (145). They commented on the instability of the gas vesicle-containing phenotype, which was lost with a frequency as high as 1%. The loss of gas vesicles was shown by Simon to be correlated with loss of a plasmid (193). Subsequent studies indicated that some gas vesicles were produced in late exponential phase by the so-called gas-vesicle-deficient mutants, but they were of a different morphology and contained a protein that had a slightly different electrophoretic mobility from those of the wild type (194). Gas-vesicle-deficient mutants were also found in *Halobacterium halobium* (207). This species contained a 100-MDa (150-kbp) plasmid, which was modified by insertion elements in the mutants (271). This suggested that the plasmid carried genes that determined or controlled gas vesicle formation. The wild-type and mutant phenotypes were described as being $Vac^+$ (gas vacuole) and $Vac^-$, respectively; the gene interrupted by these insertions was therefore termed *vac* (174). A cluster of 13 (80, 106) or 14 (59, 91) genes involved in gas vesicle production has now been located on this plasmid.

***Halobacterium* GvpA.** By using N-terminal amino acid sequence analysis of intact gas vesicles isolated from *Halobacterium halobium*, we demonstrated a protein that was clearly homologous with the cyanobacterial GvpA (226) (Fig. 2). N-terminal sequences almost identical to that of the *H. halobium* protein were found in gas vesicles from *H. salinarium* (226) and in gas vesicles isolated from several other strains of halobacteria (209). Of particular interest was the finding by Surek et al. (209) that in five different gas-vesicle-deficient strains, which had reduced amounts of gas vesicles, the gas vesicle protein sequence differed from that of the *H. halobium* wild type at two positions, 7 (Gly→Ser) and 28 (Val→Ile) (Fig. 8).

Genes encoding the halobacterial GvpA were isolated by probing the *Halobacterium* DNA with oligonucleotides that had sequences derived from part of the *Halobacterium* GvpA amino acid sequence (209) or with a fragment of the *gvpA* gene from the cyanobacterium *Calothrix* sp. (39, 90). The halobacterium genes were designated either *gvpA* (39), because of their homology with the cyanobacterial gene (210), or *vac* (90), because they corresponded to the *vac* locus mutated by the insertion elements (174); the name *gvpA* is now used (58) for uniformity with the nomenclature in cyanobacteria.

Horne et al. (90) found two forms of *gvpA* in a strain of *Halobacterium halobium*. One, which I shall refer to as *p-gvpA* (corresponding to *p-vac* in reference 90), was carried on a 180-kbp plasmid, pFP1; it encoded a protein, p-GvpA, of 75 amino acids, whose N-terminal sequence was identical to that of the protein in the gas vesicles isolated from the wild-type strains (209). The second gene, *c-gvpA* (*c-vac* [90]), was carried on the chromosome; it encoded a protein, c-GvpA, of 78 amino acids, whose N-terminal sequence was identical to that of the protein in the gas vesicles isolated from the gas-vesicle-deficient strains (209). Comparison of the deduced amino acid sequences indicated that, apart from the changes at residues 7 and 28 already referred to, p-GvpA otherwise differed from c-GvpA in lacking 3 residues, Pro-Glu-Pro, near the C terminus (Fig. 8). Northern (RNA) hybridization studies showed that in *H. halobium* the *p-gvpA* gene was expressed most strongly during the exponential growth phase of a culture, whereas transcription from the *c-gvpA* occurred only during the stationary phase of growth (92). The plasmid gene was found to be highly susceptible to loss through the combined effects of deletion events and the integration of insertion elements (172). Such mutants produced very few gas vesicles in the exponential growth phase, but they gradually accumulated

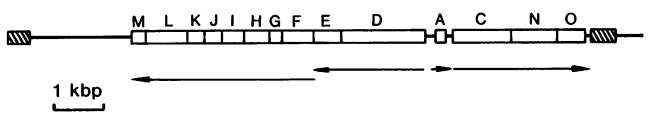


FIG. 9. Organization of the *p-gvp* gene cluster in *Halobacterium halobium* showing the 14 ORFs, the location of the flanking insertion elements ISH27 (left) and ISH2 (right), and the size and orientation of the four mRNA transcripts. The position of *gvpO* is inferred from that in the homologous gene cluster in *Haloferax mediterranei* (58). Modified from Horne et al. (91) with permission from the publisher.

gas vesicles in the stationary phase. This discovery of the two similar but distinguishable protein products of the genes carried on the plasmid and the chromosome explained the curious behavior of the gas-vesicle-deficient mutants. The small differences in the amino acid sequence of p-GvpA and c-GvpA may be responsible for differences in the shape of the gas vesicles produced by the wild-type and mutant halobacteria (see Morphology of Gas Vesicles, below).

Separate chromosomal *c-gvpA* and plasmid *p-gvpA* genes have been found in another strain of *H. halobium* that bears a 0.2-Mbp plasmid, pNRC100 (106), and also in *H. salinarium* (58). In each of these organisms the amino acid sequences of the p-GvpA and the c-GvpA they encode are the same as those in *H. halobium* (Fig. 8). In *Haloferax mediterranei* there is only a single, chromosomal gene, which encodes an amino acid sequence nearly identical to that of c-GvpA in *H. halobium*; the N-terminal residue is Val instead of Ala, and near the C-terminus it shows two differences from c-GvpA of *H. halobium* (Fig. 8).

Samples of gas vesicles isolated from halobacteria (*H. salinarium* and *H. halobium*) subjected to electrophoresis in phenol-acetic acid-urea (PAU)-polyacrylamide gels gave only a single mobile band (194, 209). It was suspected in each case that the band was formed by the constituent GvpA. In each species the mobility of the band obtained with the wild type, which expresses the 75-residue p-GvpA, was slightly higher than that of gas-vesicle-deficient mutant, which expresses the larger 78-residue p-GvpA. Nevertheless, the identities of the proteins in these bands were uncertain because the mobilities indicated molar masses, in the region of 17 to 18 kDa (194) or 19 to 20 kDa (209), that were considerably larger than those indicated by the GvpA amino acid sequences and did not correspond to those of any of the other *gvp* gene products (see Table 1). Halladay et al. (80) have now confirmed by immunoblotting procedures that the bands comprise 8-kDa GvpAs, which run anomalously in the PAU-polyacrylamide gels. It can be concluded, therefore, that the gas vesicles produced by the wild types contain the p-GvpA and that those produced by the mutants contain the c-GvpA.

**Gas vesicle proteins encoded by other genes.** The involvement of other genes in the process of gas vesicle formation in halobacteria was suggested by the finding that gas-vesicle-deficient mutants of *Halobacterium halobium* contained insertion elements incorporated into the plasmid both upstream (105) and downstream (91, 106) of the *gvpA* gene. In all, 13 (80, 106) or 14 (58, 91) contiguous ORFs on a 9-kbp region of the plasmid have been implicated in gas vesicle formation by such observations. The organization of these ORFs was found to be highly compact, with, in some cases, the end of one reading frame overlapping the beginning of the next; the arrangement with respect to *p-gvpA* is shown in Fig. 9. Three of the genes (*p-gvpC*, *p-gvpN*, and *p-gvpO*) are located downstream of *p-gvpA* on the same DNA strand, while the other 10 (*p-gvpD*, *p-gvpE*, *p-gvpF*, *p-gvpG*, *p-gvpH*, *p-gvpI*, *p-gvpJ*, *p-gvpK*, *p-gvpL*, and *p-gvpM*) were located upstream on the other strand in the opposite transcriptional orientation. Extensive sequencing has shown that the organization of the 9-kbp region is essentially similar on plasmids pHH1 (91) and pNRC100 (106), from two different strains of *H. halobium*, and on the chromosomes of *H. salinarium* and *Haloferax mediterranei* (58). In both of the plasmids the 9-kbp region is flanked by two insertion elements in inverted orientation, which suggests that it might behave as a composite transposon (106). Englert et al. (58) showed that the nucleotide sequences of the 14 genes on the plasmid of *H. salinarium* are less closely related to those on the chromosome of the same organism than to those on the chromosome of *Haloferax mediterranei*. This provides evidence that there has been lateral transfer of these clusters between organisms.

Transcripts from the 9-kbp region of the *H. halobium* plasmid have been identified by Northern hybridization, and their start sites have been determined by S1 nuclease protection studies (91). Four transcripts were located, encompassing *gvpA* and *gvpC–N* on one strand and *gvpD–E* and *gvpF–M* on the other strand (Fig. 9). In *Haloferax mediterranei* four types of transcripts are produced from a common promoter upstream of *gvpA*, encompassing *gvpA*, *gvpA/C*, *gvpA/C/N*, and *gvpA/C/N/O*; most of the transcripts terminate 64 bp downstream of *gvpA* (59). In *Haloferax mediterranei* and *Halobacterium salinarium* the transcripts of *gvpF–M* were produced mainly in the logarithmic phase of growth whereas the others were produced mainly in the stationary phase (58). This may indicate that the gene products of *gvpF–M* are required to establish the conditions for gas vesicle production and perhaps to form the initial structures from which the gas vesicles are assembled (173).

Pfeifer and coworkers (58, 91, 173) proved that all 14 of these genes are involved in gas vesicle production by showing that a strain of *Haloferax volcanii* without gas vesicles gained the ability to produce them after it had been transformed by using vectors (14) carrying restriction fragments of either the *H. halobium* plasmid or *Haloferax mediterranei* chromosome bearing the entire 9-kbp region; transformants bearing only part of the 9-kbp region failed to make gas vesicles (58, 91). Halladay et al. performed similar experiments with *H. halobium*: a mutant that had had the entire gas vesicle gene cluster deleted from the plasmid recovered the ability to make gas vesicles when transformed with a 9-kbp fragment containing only *gvpD–M* and *gvpA–N* (80).

Data on the predicted products of the 14 ORFs are given in Table 1. At present there is no direct evidence about the role of any of the gene products apart from GvpA, but the following observations have been made for some of them.

**(i) GvpC.** Downstream from the *p-gvpA* (*p-vac*) gene on the plasmid of *H. halobium* is a gene (*p-gvpC*) that encodes a protein that is very likely to be the counterpart of the cyanobacterial GvpC (91, 106). The inferred amino acid sequence indicates that after an N-terminal sequence of 21 residues there are 7 sequences of between 32 and 41 residues (39, 32, 38, 38, 32, 40, and 41 residues) that show regions of homology with one another, and then 101 amino acids to the C terminus (Fig. 10); the total number of residues is 382, and the $M_r$ is 42,391. Apart from its repeating structure, the protein also resembles cyanobacterial GvpC in the relatively high proportion of phenylalanine and of aspartic and glutamic acids or their amides. There appears to be some sequence homology between the repeating sections of the halobacterial and cyanobacterial proteins (106) (Fig. 10), although the significance of this has been questioned (59). It has recently been demon-

TABLE 1. Predicted products of *gvp* genes from *Halobacterium halobium*, *Halobacterium salinarium*, and *Haloferax mediterranei*[a]

| Gene product | Approx mol mass (kDa) | No. of amino acids[b] in: | | | | Comments |
|---|---|---|---|---|---|---|
| | | *H. halobium* plasmid gene | *H. salinarium* plasmid gene | *H. salinarium* chromosomal gene | *H. mediterranei* chromosomal gene | |
| Strand 1 | | | | | | |
| GvpA | 8.1 | 75 | 75 | 78 | 77 | Principal component; forms ribs[c] |
| GvpC | 42.4 | 382 | 382 | 385 | 381 | Outer protein, stabilizes structure[c] |
| GvpN | 39.2 | 347 | 346 | 345 | 347 | Has a nucleotide binding site |
| GvpO | ND[d] | ? | 120 | 111 | 140 | |
| Strand 2 | | | | | | |
| GvpD | 59.3 | 536 | 536 | 492 | 545 | Has a nucleotide binding site, regulates expression[e] |
| GvpE | 21.0 | 191 | 191 | 192 | 192 | |
| GvpF | 24.0 | 213 | 214 | 217 | 213 | |
| GvpG | 10.0 | 83 | 83 | 83 | 83 | |
| GvpH | 19.9 | 182 | 182 | 163 | 216 | |
| GvpI | 16.3 | 144 | 140 | 114 | 162 | A basic protein |
| GvpJ | 12.0 | 114 | 114 | 98 | 114 | Partially homologous with GvpA and GvpM |
| GvpK | 12.7 | 113 | 113 | 119 | 117 | |
| GvpL | 32.0 | 281 | 281 | 273 | 322 | |
| GvpM | 9.2 | 84 | 84 | 73 | 86 | Partially homologous with GvpA and GvpJ |

[a] Data from Jones et al. (106) and Halladay et al. (80) for *H. halobium* and from Englert et al. (58) and Horne et al. (91) for *H. salinarium* and *H. mediterranei*.
[b] Including the N-terminal Met, except for GvpA.
[c] By analogy with the function demonstrated in cyanobacteria.
[d] ND, not determined.
[e] From Englert et al. (60).

strated by immunoblotting with antibodies raised against either recombinant *Halobacterium* GvpC or a LacZ-GvpC fusion protein that GvpC is present in gas vesicles isolated from *Haloferax mediterranei* (59) and *Halobacterium halobium* (80).

**(ii) GvpJ and GvpM.** The products of two of the genes, *gvpJ* and *gvpM*, show homologies with GvpA (Fig. 8). GvpJ would be 110 amino acids long: comparing the overlapping sequences, 32% of the residues are identical to those in halobacterial p-GvpA and 36% are identical to either the cyanobacterial consensus sequence of GvpA or the GvpA in *Dactylococcopsis salina*. GvpM would be 83 residues long, of which only 15% are identical to halobacterial GvpA and 24% are identical to cyanobacterial GvpA; 33% of the residues are identical to those in GvpJ. Although these overall percentages are not particularly high, GvpJ and GvpM contain stretches of sequence between residues 10 and 60 that show much higher homology (Fig. 8) and confirm these products as part of a GvpA family. It is therefore likely that these proteins have a structural role in the gas vesicle. We can only guess what that might be. Do they influence shape? Are they components of the cone (106)? Do they initiate the assembly? These proteins may be present in such small quantities that they escape detection, but they might conceivably be located by antibody-labeling techniques (59, 78, 80, 180).

**(iii) GvpD.** *gvpD* , the first ORF upstream from *gvpA*, encodes the largest of the putative proteins, of 536 to 545 amino acids in the different organisms (Table 1). Englert et al. (60) produced transformants of *Haloferax volcannii*, ΔD, in which part of the *gvpD* gene had been removed (such that its product was a shorter protein but with the same N- and C-terminal amino acid sequences). The cells of ΔD produced such large numbers of gas vesicles that they became inflated by them. Evidently *gvpD* has a role in regulating the overexpression of gas vesicle proteins. Jones et al. (105) have pointed out

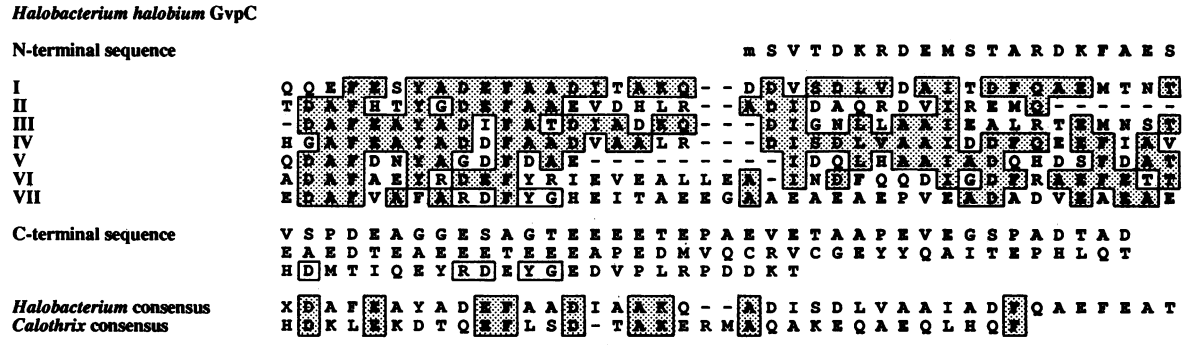


FIG. 10. Amino acid sequence of GvpC from *Halobacterium halobium* inferred from the nucleotide sequence of *gvpC*. The N-terminal sequence of 19 (or 20) residues is followed by the seven partially conserved repeats of 32 to 41 residues and then the C-terminal sequence of 100 residues; the end of the C-terminal sequence is aligned so as to show the homology of certain residues (boxed but unshaded) with those in the seventh repeat. (Data from Horne et al. [91] and Jones et al. [106].) The last two rows compare the consensus sequence of the seven repeats with that of the four repeats in GvpC from *Calothrix* sp. (37). Identical residues are boxed and shaded.

that part of the inferred amino acid sequence of *H. halobium* GvpD, L-I-N-G-A-P-G-T-G-K-T, shows homology to nucleotide-binding sites in adenylate kinase (214) and other proteins. (A similar sequence, L-I-G-P-T-G-C-G-K-T, occurs in GvpN [80]). It has been speculated that GvpD may have a role in an energy-requiring process in gas vesicle assembly (105) or in the phosphorylation of a gas vesicle protein (91). Gas vesicle preparations from *H. halobium* have been found to contain 2% phosphate and also 1% D-galactose (134), but these substances might have come from impurities left by the rather simple purification procedure that was used.

## CRYSTALLINE STRUCTURE OF THE GAS VESICLE WALL

We know, from the foregoing, that the gas vesicle has a ribbed construction and that it is formed from (at least) two types of gas vesicle protein. What is needed now is a complete crystallographic structure of the proteins and then an analysis of how they interact to form the gas vesicle. It has not been possible to obtain this information, because the main protein (GvpA) cannot be dissolved (without being denatured), let alone crystallized for X-ray diffraction analysis. Some information on the molecular structure of the gas vesicle has been obtained by X-ray crystallography of partially oriented gas vesicles isolated from cyanobacteria (15) and halobacteria (16). Figure 11 (see also Fig. 12) shows possible interpretations of these data, aided by knowledge of the size and sequences of the constituent gas vesicle proteins and by some guesswork on how the two proteins might interact.

### Wall Thickness

The first estimates of the thickness of the gas vesicle wall, 2.8 nm, were based on electron-microscopic measurements of the lengths of shadows cast by metal-shadowed collapsed gas vesicles (108). This value must exceed the wall thickness because it includes the (unknown) thickness of the metal layer itself (231).

Blaurock and coworkers performed X-ray crystallography on layers of collapsed gas vesicles, dried onto a flat surface and oriented edge-on to the X-ray beam; a strong reflection normal to the plane of the membranes indicated a periodicity with an integer multiple of 2.0 nm (i.e., 2 nm if the reflection indicated a first-order diffraction, 4 nm for a second-order diffraction and so on) in *Halobacterium halobium* (16) and 1.95 nm in *Anabaena flos-aquae* (15). The interpretation favored was that the 2.0-nm reflection was generated by a single thickness of the gas vesicle wall, because this value was within the upper limit suggested by electron microscopy.

An independent estimate of the average thickness of the wall was obtained by determining the volume of gas space enclosed by a given volume of wall protein (253). In the *Anabaena* gas vesicle, 0.76 $mm^3$ of protein (1 mg of density 1,320 kg $m^{-3}$) enclosed 7.67 $mm^3$ of gas space. From electron-microscopic measurements of the shape and size of the gas vesicles, the average thickness, $t$, of the wall could then be calculated (253); improved measurements (86, 254) gave $t$ = 1.80 nm as the average thickness, which is slightly less than the stacking periodicity of the collapsed structures, 1.95 nm in this species (15). It is uncertain whether the gas vesicles used in either of these analyses contained the surface covering of GvpC; the estimates of stacking periodicity and average thickness may therefore be for the GvpA wall structure alone.

X-ray diffraction showed that within the plane of the *Anabaena* gas vesicle wall were two peaks of electron density 1.0 nm apart (0.9 nm in *Halobacterium* spp.), which is consistent with there being two layers of polypeptide chains within the wall; sharp reflections at a Bragg spacing of 0.47 to 0.49 nm were indicative of the dipeptide repeats in β-sheet that extended long distances within at least one of the wall layers (15, 16). The electron density of the wall material at the inner (gas-facing) surface was lower than that at the outer (water-facing) surface. This would be consistent with an abundance of aliphatic amino acid side chains rich in C and H at the inside and a relative abundance of the more electron-dense N- and O-containing side chains at the outer surface.

### Periodicities of the Ribs

Electron micrographs of gas vesicles show that both the central cylinders and the conical end caps have a regular, ribbed construction (20, 107). The ribs appear both on the inside of the structure, as revealed by freeze-fracturing (107, 224), and on the outside, as revealed by freeze-etching (223a), negative staining (257), and metal shadowing of dried collapsed gas vesicles (108). The periodicity of the ribs indicated by the various methods was between 4 and 5 nm (231). Claims were made for particles of about 3 nm along the ribs, but these have been discounted as the granular artifacts of staining and shadowing (231).

When concentrated suspensions of intact *Anabaena* gas vesicles were dried between glass points, they formed a short thread in which most of the gas vesicles were aligned with their long axes within ±22° of the thread axis. X-ray diffraction patterns of these threads, mounted perpendicularly across the X-ray beam, contained a number of sharp arcs, which could be attributed to regular molecular structure within the plane of the wall. After correcting for the imperfect alignment of the gas vesicles, it was found that the reflections formed a regular lattice. The most prominent reflection was on the principal axis and had a Bragg spacing of 4.57 nm, which was clearly generated by the ribs (15).

### Periodicities along the Ribs

Perpendicular to the principal axis of the gas vesicles was a sharp reflection in the X-ray diffraction pattern, indicating subunits regularly spaced along the ribs at intervals of 1.15 nm (15), which would not have been resolved in electron micrographs. The structure that generated this periodicity was indicated by another prominent reflection oriented at an angle of 35° to the rib axis, with a Bragg spacing of 0.47 nm. This is characteristic of the perpendicular distance between adjacent chains in β-sheet; 35° was therefore the orientation of hydrogen bonds between adjacent β-chains that ran at the complementary angle of 55° to the rib axis (Fig. 11). Assuming adjacent chains to be antiparallel (the arrangement in which all the hydrogen bonds are perpendicular to the chain axis), the repeat distance to the next parallel chain would be 2 × 0.47 nm = 0.94 nm at 35° to the rib axis and the repeat distance along the rib axis would be 0.94 nm/cos35° = 1.15 nm, as observed. These values correspond closely to the precise periodicities generated by crystalline β-sheet (15), as discussed below.

### The Subunit

These three fundamental periodicities in directions across the wall, across the rib, and along the rib defined a unit cell of volume 1.95 nm × 4.57 nm × 1.15 nm = 10.25 $nm^3$ (15). From an amino acid analysis of the protein in *Anabaena* gas vesicles (61), the density of the protein was calculated (32) as ρ = 1,320

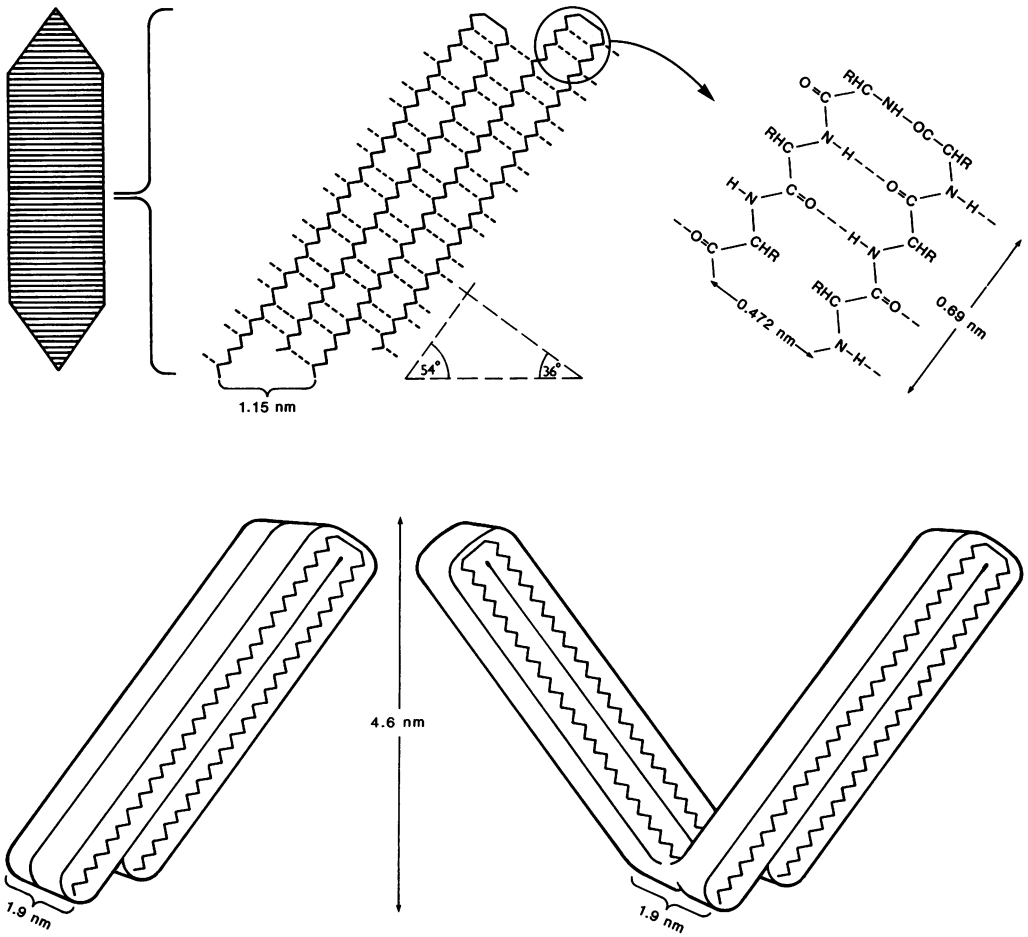


FIG. 11. Possible arrangement of GvpA molecules in the rib of the gas vesicle, based on X-ray crystallographic data of Blaurock and Walsby (15). Adjacent β-chains (of length 8 and 7 dipeptides) lie at a unique angle, α, to the rib axis. In the right-angled triangle shown, one side is proportional to the distance between pairs of chains ($2a$ = 0.994 nm) and the other side is proportional to the dipeptide repeat ($2h$ = 0.69 nm); the angle α is therefore given by $\tan^{-1}(2a/2h)$ = 53.8°; the hydrogen bonds perpendicular to the β-chains lie at the complementary angle of β = 36.2° to the rib axis (53.8° to the cylinder axis). The chirality of the structure could be opposite to that shown, i.e., with the chains sloping to the left instead of right. Modified from Walsby (251) with permission from the publisher.

kg $m^{-3}$. Multiplying by this and the Avogadro number gives the molar mass of the subunit, 8,150 g $mol^{-1}$. A slightly smaller volume (9.46 $nm^3$) and molar mass (7,500 g $mol^{-1}$) are obtained if the average thickness (1.80 nm) of the gas vesicle wall is used instead of the stacking periodicity, which may include some unoccupied space between layers.

At the time these calculations were made, the size of the gas vesicle proteins was unknown, but subsequent determination of the amino acid sequence of the *Anabaena* GvpA (87, 226) revealed a molecular mass, 7,397 Da, close to that of the unit cell. This, combined with the fact that GvpA accounted for the majority of the protein present in the gas vesicle, supported the conclusion that GvpA formed the ribs of the structure.

## Speculations on the Structure of GvpA

The foregoing is a synopsis of the structure that is supported by hard evidence. In this section I discuss the speculations for which there is, as yet, no evidence. In this way I hope to keep fact separated from conjecture.

**Antiparallel β-sheet.** The crystallographic data support the model of antiparallel β-sheet in which the hydrogen bonds lie at the same angle. The data do not preclude parallel adjacent chains, however, in which the alternate hydrogen bonds are at slightly different angles.

**Extent of β-sheet.** The sharpness of the reflections from the 0.47-nm periodicities indicates that the β-sheet extends for long distances (15). It has been proposed but not proved that alternate β-chains extend across the full width of the rib and are connected to the adjacent chains with a β-turn. This would explain the antiparallel direction of adjacent chains (Fig. 11).

**Explanation for the angle of the β-sheet.** The orientation of the β-chains can be explained by an arrangement in which the adjacent antiparallel β-chains differ in length by one dipeptide (Fig. 11). Adjacent pairs of chains would then be tilted at a unique angle to the rib axis, given by

$$\alpha = \tan^{-1}(2a/2h) = 53.8° \quad (1)$$

where $a$ = 0.472 nm, the perpendicular distance between β-chains, and $2h$ = 0.69 nm, the dipeptide repeat in the chain. The H bonds will therefore lie at the complementary angle of 36.2° to the rib axis (53.8° to the cylinder axis). This angle is within 1.2° of the measured value. Both the calculated angle α and the observed angle are very close to the "magic" angle of 54.7°, at which the transverse and longitudinal stresses are

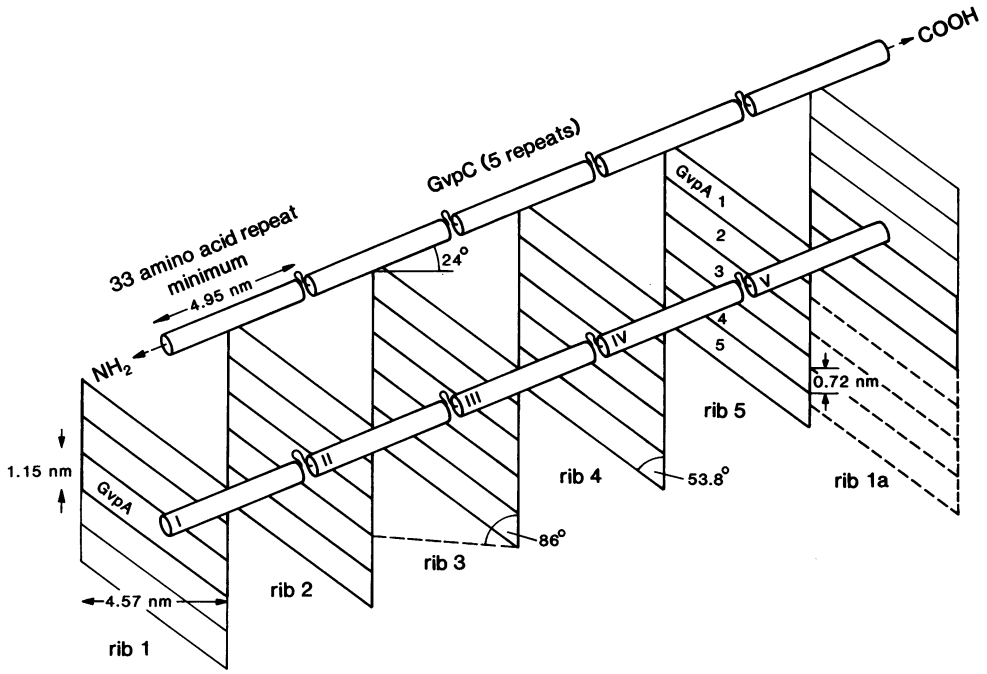


FIG. 12. Speculations on how GvpC might interact with GvpA in the ribs. GvpA molecules are stacked like rhomboidal bricks at intervals of 1.15 nm along the ribs (Fig. 11). A line joining the equivalent points of GvpA molecules in adjacent ribs forms an angle of 86° to the rib (data of Blaurock and Walsby [15]), so that at the rib junctions the GvpA molecules are offset by 0.72 nm. GvpC molecules comprise five 33-residue repeats (84) that in α-helical conformation would each have a minimum length of 5.0 nm (the length of 9.2 gyres, each of 3.6 residues and length 0.54 nm). Each repeat could contact five GvpA molecules in crossing the 4.57-nm-wide ribs at an angle of 24°, so that the five repeats would contact 25 GvpA molecules; this might generate the observed GvpA/GvpC ratio of 25:1. Secondary-structure predictions suggest that a few residues (around the sequence Q-F-L, see Fig. 5) in each of the 33-residue repeats would break the helix and increase the overall length slightly.

equal in the wall of a cylindrical structure (see below). As discussed below (see Modifications of GvpA), there are reasons for believing that natural selection for gas vesicles of maximum strength has favored the evolution of an amino acid sequence that caused the peptide chains to fold at this angle.

**Length of the β-chains.** The length of a β-chain oriented diagonally across a rib would be 4.57 nm/cos36.2° = 5.66 nm. This length would accommodate a chain of 8 dipeptides (8 × 0.69 nm = 5.52 nm). It is therefore proposed that the adjacent chains alternate in length between 8 and 7 dipeptides. Since there are two layers of β-sheet, the two pairs of chains in each unit cell would contain 30 dipeptides, or 60 of the 70 amino acid residues in GvpA. This would leave a further 10 residues to be accommodated above or below the two layers of β-sheet.

**Direction of β-chains in each layer.** It is not possible to tell whether the β-chains slope to the right (i.e., at an angle of +54° to the rib axis, as depicted in Fig. 11) or to the left (at −54°) because the same structure that appears to an observer (or in this case the X-ray beam) to slope right on the front face of a cylinder appears, when viewed through the cylinder, to slope left on the back face. Similarly, it is not possible to tell whether the β-chains slope in the same or opposite direction within the two layers of β-sheet in the wall: the X-ray diffraction pattern from a cylindrical structure is always identical in the four quadrants. (We have just resolved one of these problems: by atomic force microscopy we have obtained images that show that in the outer layer the β-chains slope to the left, i.e., opposite to the direction depicted in Fig. 11 [151a].)

**Other unresolved features.** It has been calculated that if the β-chains in the two layers ran in the same direction, the ridges formed by the amino acid residues in the upper layer would fit into the grooves between the ridges in the lower layer, forming a tightly interlocked structure (15). On the other hand, it can be postulated that the β-chains should run in opposite senses to prevent the structure from twisting under pressure.

There are several features of the gas vesicle that are not accommodated by the simple model represented in Fig. 11. First, analysis of the gas vesicle proteins by circular dichroism indicates the presence of some random coil and α-helix as well as the β-sheet confirmed by X-ray diffraction. Only a small proportion of α-helix would have been accounted for by GvpC if it had been present. Second, analysis of the amino acid sequence of GvpA by programs for secondary-structure prediction (45) suggest that only part of the polypeptide chain is in the form of β-sheet. Third, electron microscopy of metal-shadowed or negatively stained gas vesicles indicates that the ribs form corrugations on both surfaces of the wall, whereas the model suggests a planar surface, although it is possible that part of the unevenness of thickness is generated by the 10 residues of each GvpA molecule that must lie outside the β-sheet.

### Speculations on How GvpC Binds to Gas Vesicle Ribs

We have suggested that the GvpC molecules might form ties that bind the ribs of GvpA together. Secondary-structure predictions (45) indicate that the 33-amino-acid repeats have an α-helical structure. An α-helix of 33 amino acid residues would contain 9.2 gyres each of length 0.54 nm, giving an overall length of 5.0 nm (84). This slightly exceeds the width of each rib, 4.57 nm. Hence, as explained in Fig. 12, the first of the 33 amino acid repeats could cross the entire width of the rib at

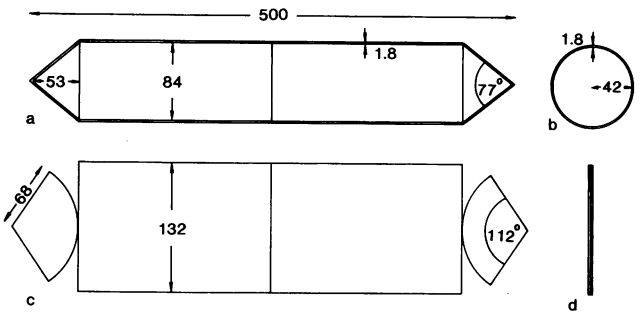


FIG. 13. Geometry of the *Anabaena* gas vesicle drawn to scale, with all measurements in nanometers; (a) intact gas vesicle longitudinal half, with double lines representing the wall thickness in the plain of the section; (b) cross-section of panel a; (c) collapsed gas vesicle, face view; (d) cross-section of panel c. The gas vesicle may be formed from two identical halves joined back-to-back at the center line. Reproduced from reference 251 with permission from the publisher.

an angle of 24°, making contact with parts of five GvpA molecules, each of which has a periodicity of 1.15 nm along the rib. Because the next 33-residue repeat has a similar amino acid sequence, it would be able to make almost identical contacts with five GvpA molecules in the adjacent rib; the third, fourth, and fifth repeats would form similar attachments, linking five adjacent ribs. According to this model, the GvpC molecule will contact five GvpA molecules in each of five ribs, giving a GvpA/GvpC ratio of 25:1. This corresponds to the observed ratio in isolated gas vesicles and to the amount of GvpC that gas vesicles will readsorb after they have been stripped of their native GvpC (22). Other models are discussed elsewhere (22).

If GvpC does form this sort of arrangement, I would guess that the beginning of each 33-amino-acid repeat does not bind at the edge of the rib, so that the repeat is confined within a single rib but rather that it starts partway across a rib and overlaps to the adjacent rib (Fig. 12). In this way a molecule with five repeats would bind six ribs. One can more easily envisage the evolution of such an arrangement from a cell protein that binds across two adjacent ribs and thereby stabilizes the structure.

## MORPHOLOGY OF GAS VESICLES

In this section I describe the morphology of the gas vesicle, how it forms, and which features of the gas vesicle proteins may control its size and shape, which together have a profound effect on its efficiency in providing buoyancy and its strength in resisting collapse.

### Description of Gas Vesicle Morphology

Gas vesicles in all species of cyanobacteria investigated have the form of hollow cylinders with conical end caps. In each species the width of the cylinders is fairly uniform, but, partly as a consequence of the way in which gas vesicles form (see How the Gas Vesicle Forms and Grows, below), their length varies from 100 to 800 nm or even more. Gas vesicles of the same basic morphology, but a wider range of size, are found in other groups of bacteria and archaea.

**Gas vesicle width.** The cylinder diameter ($d$) of gas vesicles can be accurately determined from measurements of the widths ($w$) of metal-shadowed collapsed gas vesicles, using electron micrographs calibrated with diffraction gratings: $d = 2w/\pi$ (108, 254) (Fig. 13). In different freshwater cyanobacteria, $d$ varies from 62 nm in red-pigmented *Oscillatoria agardhii* to 84 nm in *Anabaena flos-aquae* (86, 254). Wider gas vesicles were found in two halophilic cyanobacteria, 109 nm in *Dactylococcopsis salina* (254) and 117 nm in *Aphanothece halophytica* (110a). The narrowest gas vesicles in cyanobacteria, approximately 45 nm in cylinder diameter, have been reported in the oceanic species *Trichodesmium thiebautii* (69).

Within each species of cyanobacterium the cylinder diameter is fairly uniform, with a standard deviation of about ±4%. This value must include small measurement errors, but there are genuine differences in the widths of individual gas vesicles: gas vesicles of different strengths in *Anabaena flos-aquae* were separated after application of different pressures, and it was found that the weakest 10% had a mean diameter 6.2 nm wider than the strongest 10% (254). The range of widths indicates that the number of GvpA molecules in a rib varies by ±9 about a mean of 229 GvpAs; evidently, the rib is not assembled with molecular precision.

Gas vesicles with a wide range of size and shape are also found in other microorganisms. Long, narrow gas vesicles, resembling those in freshwater cyanobacteria, occur in the green sulfur bacterium *Pelodictyon clathratiforme* (175), in a number of other unnamed bacteria (89, 205), and in the archaea *Methanosarcina* sp. (279), *Methanosarcina barkeri* (6), *Haloferax mediterranei* (57), and the halophilic square bacterium (169, 240). A number of other microorganisms, including the purple photosynthetic bacteria *Amoebobacter bacillosus* (31), *Amoebobacter rosea* (231), and *Thiodictyon elegans* (31) and the colorless bacteria *Prosthecomicrobium pneumaticum* (202, 238), *Ancalomicrobium adetum* (202), *Aquabacter spiritensis* (99), and *Ancylobacter* (*Microcyclus*) *aquaticus* (89, 219), have gas vesicles that are generally shorter and wider, like those in the halophilic cyanobacteria. In none of these organisms, however, have the precise dimensions been determined.

**Gas vesicle end caps.** The end caps of the cylindrical gas vesicles are invariably described as hollow cones. Straight-sided cones are indicated in electron micrographs of some freeze-fractured gas vesicles (Fig. 14) (238) and by the forms of collapsed gas vesicles (108). (A cone collapses to a sector of a circle that has a radius equal to $s$, the side of the cone.) The end caps of negatively stained intact gas vesicles usually appear to have more rounded sides; this appearance probably results from the partial sagging of the cylinder, which is indicated by its greater width in negatively stained specimens. Some gas vesicles appear to end in a small point or tail (6, 231). There is a lack of information on the shape of the cone and the uniformity of its length and end angle (see 108).

The majority of gas vesicles in *Halobacterium halobium* and *H. salinarium* have the form of a wide, rounded bicone, without a cylindrical midsection; they are described as being lemon shaped or spindle shaped. These gas vesicles may have widths of 200 nm or more (145, 207). The same organisms also produce some gas vesicles that are longer, narrower, and of cylindrical form like those in other halophilic archaea. As already discussed, the two morphotypes appear to be regulated by different gas vesicle gene clusters, one on a plasmid and the other on the chromosome (194, 209). The possibility that the morphological differences are determined by just a few changes in the GvpA amino acid sequence is discussed below (see What Determines Diameter and Length of the Gas Vesicle?). A mutant of *Ancylobacter* (*Microcyclus*) *aquaticus* that makes only the biconical gas vesicles has been isolated (133).

The general conclusion is that the gas vesicles in all of the bacteria and archaea are of the same basic form but vary in dimensions.

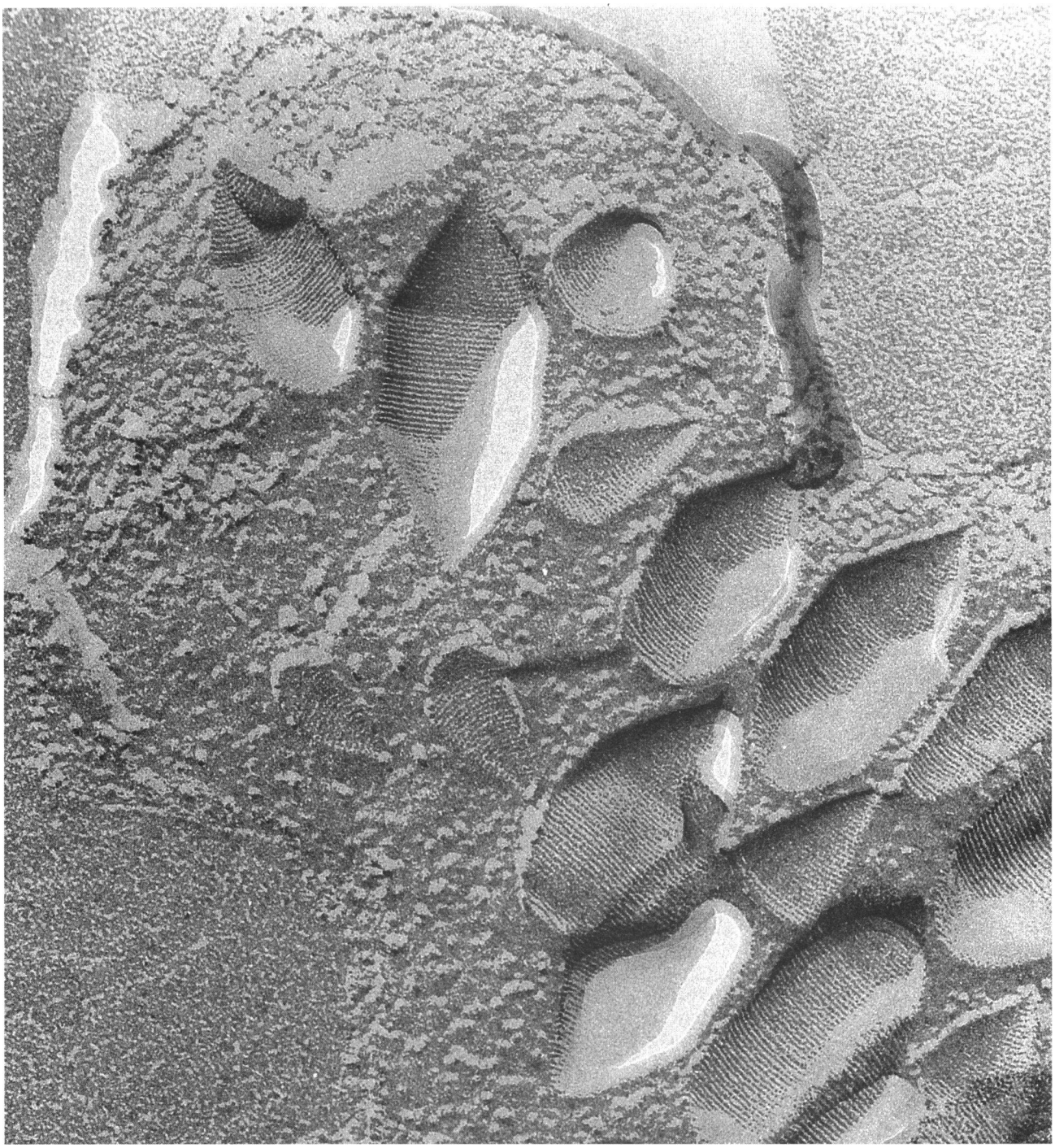

FIG. 14. Gas vesicles in a freeze-fractured cell of *Prosthecomicrobium pneumaticum* showing the straight-sided cone, the ribs in the cone and cylinder, and the prominent central rib where the two identical halves of the structure may be joined back-to-back. Reproduced from reference 238 with permission from the publisher. (Micrograph by D. Branton and A. E. Walsby.) Magnification, ×210,000.

### How the Gas Vesicle Forms and Grows

**Growth from biconical initials.** It has been argued that since, owing to their permeability, gas vesicles cannot be inflated by gas, they must be produced by a process of self-assembly (228). In the cyanobacterium *Nostoc muscorum*, gas vesicles form de novo in hormogonia whose differentiation is induced by changing the light intensity (225) or culture medium (7, 224). From a sequence of samples taken after induction, it has been demonstrated that the gas vesicle grows from a small biconical structure that enlarges to a critical diameter and then, over several hours, gradually lengthens by the extension of the cylinder that forms between the terminal cones (224). The same sequence of events has been demonstrated in cyanobacteria (147) and aerobic bacteria (133) that form gas vesicles constitutively, after the existing gas vesicles had been destroyed by pressure. These experiments demonstrate that the collapsed

structures cannot be reerected directly, although results of radiocarbon-labeling experiments suggest that some of the gas vesicle protein is recycled (85).

**Speculations on the assembly process.** Self-assembly is a property of proteins that form biological structures. The assembly process may be a form of crystallization that is independent of other substances, it may require or be accelerated by chaperonins that form templates, and it may require an activation step. The requirement for additional factors in the assembly of gas vesicles may be revealed by analysis of the various gene products (91, 106).

The gas vesicle must form by the linear assembly of GvpA molecules to form the ribs. It is still not known, however, whether the ribs are entire hoops or turns of a shallow helix (cf. references 108 and 231). A good end view of the tip of a gas vesicle that has been freeze-etched and rotatory-shadowed is needed to settle this point. In the following analysis of gas vesicle assembly (251), it is first assumed that the ribs form a helix.

(i) Gas vesicle formation must start from two protein molecules (GvpA or one of the other *gvp* gene products) joined back-to-back. To each of these, molecules of GvpA must be added one by one and in the equivalent orientation to form an extending conical helix. It is known from antibody-labeling experiments that GvpA (or a protein with the same N-terminal sequence) occurs very close to the cone tip (180).

(ii) Successive GvpA molecules might be added at either the tip or the base of each growing conical helix. Addition at the base would require no rearrangement of the structure already formed. If proteins were to be added at the tip, the previously formed turns of the conical helix would, because of their unequal diameters, have to slip past one another. There could be no coherence between the ribs (or binding by GvpC across ribs) in the cone until after growth of the entire gas vesicle had ceased.

(iii) The two cones must grow at the same rate to maintain bases of the same circumference. This could be ensured by a mechanism that added GvpA molecules either simultaneously or alternately to each cone.

(iv) The two cones must be identical. It follows, for reasons of polarity, that the contact relationship between the ribs at the base of each cone (i.e., at the centre of the bicone) must be different from that elsewhere in the structure (231).

(v) On reaching a certain critical size, the helix must subsequently extend as a cylinder instead of a cone. It is not necessary to invoke different proteins for the cone and cylinder (238). The GvpA molecules may be bent out of their most relaxed form in the tighter coils of the cone and achieve their minimum free energy in ribs with the diameter of the cylinder (see below, What Determines Diameter and Length of the Gas Vesicle?).

(vi) Once the cylinder starts to form, growth could continue through further additions to the end of each helix at the center of the structure. The different contact relationship there could explain why a distinctive rib is often seen near (but not always exactly at) the center of the gas vesicle (108, 224, 231) (Fig. 14). Alternatively, the cylinder might grow from only one of the cones, in which case the distinctive rib would be at the cone base, where it would not be apparent. It might be possible to determine where the new protein is added by pulse-labeling experiments.

(vii) It has been shown by antibody-labeling techniques that GvpC is present on gas vesicles of all lengths isolated from *Anabaena* species, including the short gas vesicles, which must be in the early stages of growth. This indicates that GvpC binds to the available sites on the GvpA molecules as they assemble to form the structure; it would therefore provide the essential stiffening as the structure grows (22).

(viii) If the ribs were hoops instead of gyres of a helix, formation would be more complex in a number of respects. Initiation might necessitate a small ring of GvpA molecules. Two additional events would be needed during extension, the joining of the last protein in the hoop to the first one, and the initiation of the next hoop (251). A gap would be present in each hoop until it was completed. Growth by addition of GvpA molecules at the cone tips would not be possible [see (ii)].

(ix) It has been proposed that the hollow space is formed within the initial aggregate of GvpA molecules by the way in which new GvpA molecules are inserted (236). The force required for expansion of the hollow structure would be provided by the decrease in free energy that occurs when the hydrophobic surface of the GvpA molecule moves from water into the gas vesicle wall (228).

## What Determines Diameter and Length of the Gas Vesicle?

**Diameter.** The eventual width of a gas vesicle must be determined by the critical maximum width that the bicone reaches before it extends as a cylinder. This width might be determined in one of two ways, either by a template (like a round tower built to a pattern from bricks whose shape imposes no restrictions on form) or by the shape of the building block (like a spiral staircase constructed from prefabricated units).

The arrangement of the GvpA molecules might be governed by some other molecule that constrains the width of the expanding bicone. Alternatively, the critical width of the gas vesicle may be determined by the shape of the GvpA molecules themselves (238). These molecules can be conceived as building blocks with a slight curvature that, when laid end-to-end, form naturally into a circle (or, with a twist, into a shallow gyre) whose diameter is determined by the angle subtended by the end walls of the block. When inserted into the tighter circles or gyres of the growing cone, either the blocks would be bent or their ends would no longer form a flush fit together. This would cause a tension in the blocks that forces a widening of the rib until the critical width is reached.

The amino acid sequences that specify different widths of gas vesicles could be determined by analyzing the effects of site-specific mutations. For example, the GvpA amino acid sequences in *Anabaena* and *Microcystis* spp. differ in only 3 of the first 64 residues (87), but the gas vesicles in these organisms are, respectively, 84 and 65 nm wide (254). By mutating the *Anabaena* sequence at each site, separately and in combination, the diameter-determining sequence might be found.

In *Halobacterium* species the morphological differences in gas vesicles might be caused by the few differences in amino acid sequence between p-GvpA, which forms the spindle-shaped gas vesicles of the wild type, and c-GvpA, which forms the cylindrical gas vesicles of the mutant (209). If this is so, the cause of the change between spindle-shaped and cylindrical morphology is narrowed to the mutations Gly→Ser at residue 7, Val→Gly at residue 28, and the additional tripeptide Pro-Glu-Pro at residues 72 to 74 in c-GvpA of *Halobacterium halobium* (Fig. 8). One question that is unresolved about the mutant gas vesicles, however, is why only the majority, and not all of the gas vesicles, had cylindrical morphology (194). Perhaps the amino acid sequence of c-GvpA does not absolutely specify the cylindrical morphology but only increases the likelihood of formation of a gas vesicle with cylindrical rather than spindle-shaped morphology. Alternatively, the spindle-shaped gas vesicles might have been formed by revertants.

The three serine residues at positions near the N terminus may be crucial in determining cylindrical morphology; they also occur in the only GvpA of *Haloferax mediterranei*, which has cylindrical gas vesicles, and in the GvpA of the bacteria *Amoebobacter pendens* (78) and *Ancylobacter aquaticus* (21a), which have short, wide cylindrical gas vesicles. All cyanobacteria, with cylindrical gas vesicles, have four *ser* residues there (Fig. 2). The differences at the two other positions (residues 28 and 72 in Fig. 8) are not consistently correlated with either morphology.

**Length.** The gas vesicles of *Nostoc muscorum* may reach a length of 1,400 nm in 24 h (224), whereas those of *Microcystis aeruginosa* take about 12 h to grow to their maximum length of about 600 nm (147). The mean lengths of gas vesicles varies from 293 to 721 nm in seven genera of cyanobacteria (254). Those in *Anabaena flos-aquae* showed a length distribution with a standard deviation of about 35% of the mean (230). The length distribution found at any instant reflects the balance of the numbers of gas vesicles being initiated, growing, reaching their maximum length, and being destroyed by collapse (230) or other means of disintegration (148).

It is not known what causes gas vesicles to stop growing. Konopka et al. (133) found that following gas vesicle collapse in the bacterium *Ancylobacter* (*Microcyclus*) *aquaticus* there was a constant rate of gas vesicle membrane production; initially only 15 new gas vesicles were produced per cell, and no more were produced until these ones had reached the length at which they began to add their cylindrical midsections. This suggests that there may be additional requirements for cylinder elongation. Alternatively it may indicate that there is a greater tendency for new GvpA molecules to assemble into existing bicones than into new ones and an approximately equal tendency to assemble into new bicones and existing cylinders.

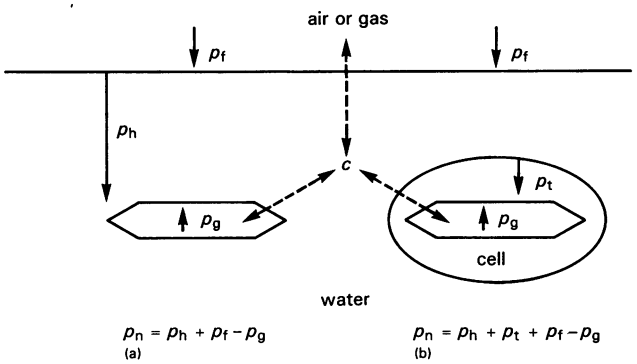


FIG. 15. Pressures acting on a gas vesicle suspended in water. (a) An isolated gas vesicle is subjected to pressures from the gas above the suspension ($p_f$) and hydrostatic pressure ($p_h$). (b) A gas vesicle inside a cell is also subjected to cell turgor pressure ($p_t$). The net pressure ($p_n$) acting across the gas vesicle wall is given by the sum of these pressures minus the gas pressure inside the gas vesicle ($p_g$); $p_g$ is determined by the concentration ($c$) of gas dissolved in the water, which is usually air at the ambient pressure (about 0.1 MPa, or 1 bar). The gas vesicle will collapse if the net pressure, $p_n$, exceeds the critical pressure, $p_c$. Modified from references 230 and 251 with permission from the publisher.

## PHYSICAL PROPERTIES OF GAS VESICLES

### A Model for the Gas Vesicle: a Pair of Flowerpots

It is unavoidable that when we consider structures in bacteria, invisible to the naked eye, we draw analogies with everyday objects that we can see and handle. Unfortunately the gas vesicle seems to evoke inappropriate comparisons, with bubbles or balloons, which are misleading and a hindrance to the understanding of the structure. To drive them out of the reader's mind, I suggest an alternative: take a pair of those old-fashioned earthenware flowerpots (but without drainage holes in the bottoms), coat the inner surfaces with a film of oil, and glue them together rim to rim. This pot is permeable to gas and therefore contains air at atmospheric pressure, although it does not need the air inside to maintain the hollow space. Now submerge the pots in water. Air continues to diffuse through the pores in the wall and can equilibrate to and fro between the enclosed gas space and the air dissolved in the surrounding medium. Liquid water will seep into the pores in the outer surface but will be held back from entering the oil-coated pores of the inner surface by surface tension. If the oil-coated pores are small enough, surface tension will even prevent water from being forced inside the chamber when a hydrostatic pressure is applied to the water. This pressure will be borne by the rigid wall with little decrease in volume. Of course, if sufficient pressure is applied, the wall will break and the gas-filled space will be lost. The flowerpot analogy is especially useful as an aid to understanding the mechanical properties of the gas vesicle, but with some of the other properties there are problems of scale. According to the laws of physics, surface tension will be much more effective in excluding liquid water from the minuscule gas vesicle than from pores of the flowerpot, and gas will fill the gas vesicle very much faster.

In the following section I explain these physical properties of the gas vesicle in more detail. Several of them involve pressures acting on the structure, and it is first necessary to define these pressures. The net pressure ($p_n$) acting on an isolated gas vesicle suspended in water is given by

$$p_n = p_h + p_f - p_g \quad (2)$$

(see Fig. 15) (230, 231). $p_h$ is the hydrostatic pressure of the water column: $p_h = z\rho g$, where $z$ is the depth (in meters) below the water surface and $\rho$ is the density of the water (998 kg $m^{-3}$ at 20°C); when $g$ is the acceleration due to gravity (9.8 m $s^{-2}$), $p_h$ rises by approximately 0.01 MPa $m^{-1}$. In a test tube in which $z$ is small, $p_h$ is insignificant; in the centrifuge, in which $g$ can be large, $p_h$ can be high and may cause collapse of gas vesicles during isolation procedures (230, 256). $p_f$ is the pressure of gas in the overlying gas phase. The atmospheric pressure is about 0.1 MPa (1 bar). $p_g$ is the gas pressure inside the gas vesicles. The gas inside is always in equilibrium with the gas dissolved in the suspending water, of concentration $c$ (245). $p_g$ is therefore usually 0.1 MPa and balances the atmospheric pressure. Note that the dissolved gas does not itself exert a pressure on the gas vesicle. If the pressure of the overlying gas, $p_f$, is raised artificially, the pressure is immediately transmitted to the gas vesicles through the suspending water, and it may collapse them. More gas will gradually diffuse into the suspension, raising $c$ and hence $p_g$, but this occurs only very slowly and usually affects $p_g$ only in gas vesicles close to the gas-water interface (see below). Inside cells there is an additional source of pressure, the cell turgor pressure (Fig. 15); it can range from 0 to 0.5 MPa in different organisms (see Gas Vesicle Collapse in Cells: Turgor Pressure, below). Surface tension at the gas vesicle surface and cell surface may generate additional pressures (230) (see Surface Properties of the Gas Vesicle, below).

### Gas Permeability of Gas Vesicles

The key to understanding the gas vesicle was the determination of how gas gets inside it: gas simply leaks inside by diffusion (228). With this discovery we were able to abandon the speculation on the identity of the gas (114), how the gas was generated, what the gas might be stored for (26), and the role of gas in inflating the gas vesicle. Instead we had to consider which gases could diffuse into the gas vesicle, how quickly they entered, whether gas vesicles could provide a gas diffusion channel, and how gas vesicles were assembled. I review the evidence on the first three questions here; the fourth has already been discussed.

**Which gases can diffuse into the gas vesicle?** I demonstrated the permeability of gas vesicles to gas by experiments performed with Warburg respirometers. Following a pressure rise, $p_1$, in the air over a gas-vacuolate suspension, the gas vesicles, containing gas spaces of volume $v$, took up a quantity of gas equal to $p_1v$. The same quantity of gas was evolved from the gas vesicles if the air pressure over the suspension was reduced by $p_1$. Similar results were obtained with nitrogen, oxygen, or argon gas (228). This showed that these gases could equilibrate freely between the gas vesicles and the water in which they were suspended, and thence to the overlying gas phase. In interpreting these results, it was assumed (correctly, as it transpired) that the gas vesicle was rigid and did not change volume significantly when the pressure was changed (244, 252) (see Rigidity and Elastic Compressibility, below).

A second proof of the gas permeability of the gas vesicles was provided by investigating the effect of changing the concentration of the dissolved gas on the pressure required to collapse gas vesicles (228, 230). Gas vesicles that collapsed at a pressure of 0.6 MPa (6 bars) when they had air at the atmospheric pressure of 0.1 MPa inside them, collapsed at a pressure of 0.5 MPa after the gas had been removed by equilibration under vacuum. (In such a state the gas vesicle becomes a vacuum vesicle). When the suspension was reequilibrated under air, air returned to gas vesicles, providing them with 0.1 MPa of internal support and their collapse pressure returned to 0.6 MPa (230).

By equilibrating suspensions of gas vesicles with gas at pressures above the ambient pressures, the pressure required to collapse them could be increased. It was observed that when the gas pressure was raised slowly over a suspension, the surface layer remained turbid while the rest of the suspension clarified (Fig. 16). The explanation for this is as follows. As the pressure of the gas was raised, it instantaneously generated an equivalent hydrostatic pressure throughout the suspension, which, when it was high enough, caused gas vesicles to collapse. At the surface of the suspension, however, gas diffused into the water and thence into the gas vesicles, so that the pressure difference across the gas vesicle walls did not become high enough to cause collapse (see Critical Pressure, below). The diffusion coefficient ($D$) of gases into water is so low (for $O_2$ and $N_2$, $D = 2 \times 10^{-9}$ $m^2$ $s^{-1}$) that little of the gas diffuses much farther than 1 mm into the suspension. (The exponential folding time, $t_e$, for the gas concentration to reach $1/e$ (i.e., 0.37) of the surface concentration at a distance $x_e$ below the surface is given by $t_e = x_e^2/4D$; hence, at a depth of 1 mm, $t_e$ exceeds 2 min).

Such gas pressure rise experiments, performed with different pure gases, showed that the *Anabaena* gas vesicles were permeable to $H_2$, $N_2$, $O_2$, $CO_2$, CO, $CH_4$, and Ar (230). The largest gas molecule that has been shown to penetrate is perfluorocyclobutane ($C_4F_8$) (243), which has a collision diameter of 0.63 nm, compared with 0.36 nm for $N_2$ (245). Pores

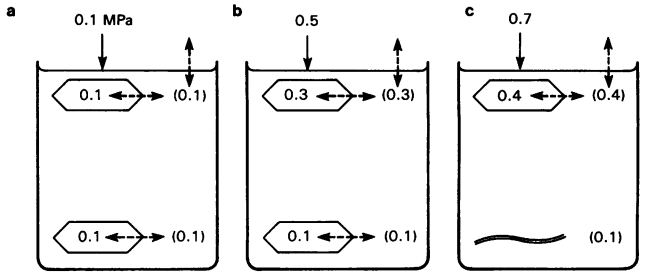


FIG. 16. Diagram showing why gas vesicles survive near the gas-water interface when the pressure of the overlying gas phase is increased above the critical pressure. (a) The suspension initially contains dissolved air at a partial pressure ($c$) of 0.1 MPa (1 bar), the pressure of the overlying air; the gas-permeable gas vesicles therefore also contain air at 0.1 MPa. (b) As the overlying gas pressure is slowly raised, gas diffuses into the surface layers of suspension and the gas vesicles there, but it does not reach the deeper layers. (c) When the pressure in the overlying gas phase reaches 0.7 MPa (0.6 MPa above the original air pressure), the net pressure ($p_n$) difference across the wall of gas vesicles deep in the solution reaches 0.6 MPa, equal to the critical pressure ($p_c$); these gas vesicles collapse. For gas vesicles containing elevated gas pressures near the surface, $p_n$ remains below $p_c$; these gas vesicles survive intact (230).

with a diameter of at least 0.63 nm must therefore occur in the gas vesicle wall. The size of pores in the structure is bound to fluctuate, however, because although the gas vesicle wall is rigid and the proteins are ordered in crystalline array, vibrations will occur in the amino acid residues that border the pores (72, 143). The time-averaged diameter of the pore could therefore be less than 0.63 nm (245).

Other gases with a collision diameter smaller than that of $C_4F_8$ must also be able to enter the gas vesicle. This probably includes all gases known to have a vapor pressure greater than 0.2 MPa (i.e., 1 bar above atmospheric pressure). It also includes the vapors of many liquids that have lower vapor pressures. Of particular interest is water, with a vapor pressure of only 0.0023 MPa at 20°C. This vapor pressure is so low that none of the methods used to prove the permeability of gas vesicles to gases can be used to demonstrate their permeability to water. The water molecule is so much smaller than $C_4F_8$, however, that there is no reason to believe that water vapor will not enter the gas vesicle.

**How quickly does gas get into the gas vesicle?** To determine the diffusion rate of gases into the gas vesicle, attempts were made to find a threshold rate of gas pressure rise that would be just sufficient to cause gas vesicle collapse, using dried films of gas vesicles to obviate the complications of diffusion through a surrounding water layer. The idea behind this experiment can be explained as follows.

Imagine a gas vesicle in an evacuated chamber (Fig. 17a). At time zero a valve connecting the chamber to a tank of compressed gas is opened and gas flows in at an exponential rate $\lambda$, recorded by a transducer, until it reaches the same pressure as in the tank, $p'$. As the pressure, $p_o$, rises in the chamber, gas starts to enter the gas vesicle, at another exponential rate $\alpha$, and the pressure inside it, $p$, also rises but, of course, always lags behind $p_o$ (Fig. 17b). The difference between the pressures outside and inside the gas vesicle is given by $p_o - p$, which reaches a maximum $\Delta p_m$ and then falls. The gas vesicle collapses if $\Delta p_m$ exceeds its critical pressure, $p_c$ (see Critical Pressure, below). A rate of pressure rise, $\lambda$, is

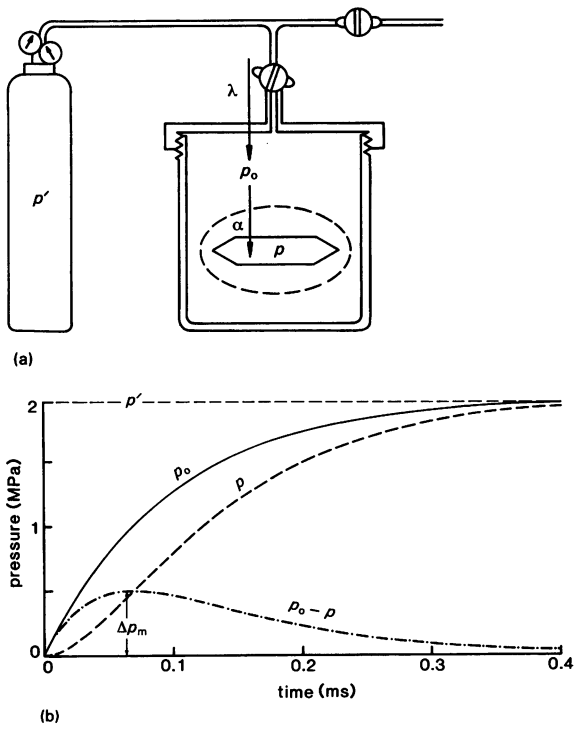


FIG. 17. (a) Representation of the method used to determine the rate at which gas fills a gas vesicle (either isolated from a cell or inside a cell [---]) enclosed in a chamber. (b) The pressure, $p_o$, in the chamber rises at the exponential rate λ to the pressure $p'$ in the tank; the pressure $p$ in the gas vesicle rises at rate α. If $p_o$ rises fast enough, the difference between $p_o$ and $p$ reaches a maximum value $\Delta p_m$ that exceeds the critical pressure, $p_c$, of the gas vesicle, which collapses; α can then be determined. Modified from Walsby (245, 246) with permission from the publisher.

found which causes half the gas vesicles in such a chamber to collapse. It is then known that $\Delta p_m = p_c$, and the only unknown, α, can be calculated. The exponential filling time of the gas vesicle, $t_e$, is the reciprocal of this; i.e., $t_e = 1/\alpha$. Details of the method are given by Walsby (245, 246).

Dried *Anabaena* gas vesicles remained intact even after the fastest pressure rise investigated (0 to 4.6 MPa [46 bar] in 2 ms). From this it could be calculated that α exceeded $22 \times 10^3$ $s^{-1}$ and that the exponential filling time, $t_e$, was less than 46 μs (245); as was later shown (264), it was nearly 100-fold less (see below, Can the Gas Vesicle Provide a Gas Diffusion Channel?). This experiment can be adapted to determine how quickly gases diffuse inside gas-vacuolate cells, which, partly because they are much larger, fill much more slowly than gas vesicles. (The method has been used to demonstrate that the filling time of cyanobacterial heterocysts with oxygen was 3.3 s, much longer than for vegetative cells [246]).

**Can the gas vesicle provide a gas diffusion channel?** It has been suggested that gas vesicles might provide gas diffusion channels (211). Are they permeable enough to do this? For gas to diffuse through a gas vesicle, it would have to pass in through the wall on one side, across the negligible resistance of the gas space, and out through the opposite wall. Could it do this faster than it would diffuse through a similar length of water? The permeability coefficient of the gas vesicle wall, κ, is related to the filling coefficient α by the expression

$$\kappa = \alpha V/A_o \quad (3)$$

where $V$ is the volume of the gas space, $2.14 \times 10^6$ $nm^3$ for the *Anabaena* gas vesicle, and $A_o$ is the area of the outer surface, $0.120 \times 10^6$ $nm^2$ (254); hence, from the lower limit of $\alpha = 22 \times 10^3$ $s^{-1}$, it can be calculated that the minimum value of κ would be 392 μm $s^{-1}$. The length, $L$, of a water layer with the same permeability as two gas vesicle walls would be $2D/\kappa$, where $D$ is the diffusivity of $O_2$ in water, 2,000 $\mu m^2$ $s^{-1}$. Substituting the minimum value of κ gives $L = 10$ μm, much longer than the length of the *Anabaena* gas vesicle (about 0.5 mm). Clearly, this minimum value of κ would not allow the gas vesicle to function as a gas diffusion channel.

What this analysis suggested, however, was that if the diffusivity of gas through a gas vesicle suspension could be measured, it would be possible to work back through these calculations and derive the permeability coefficient, κ (245). We have recently achieved this for oxygen gas by using oxygen microelectrodes (264). Stable concentration gradients of oxygen were allowed to develop across a concentrated suspension of *Microcystis* gas vesicles supported over a layer of water immobilized in agar between pure oxygen gas below and air (20% oxygen) above (Fig. 18). The gradient of oxygen concentration ($dC/dx$) was slightly steeper in the gas vesicle suspension than in the water; since the oxygen flux across the two layers would have been uniform, the diffusivity of oxygen must have been slightly lower. From measurements of the volume fraction of gas vesicles in the suspensions, it was possible to obtain an estimate of the filling coefficient, α, and from this the exponential filling time, $t_e = 0.4$ μs, and the permeability coefficient of the membrane, $\kappa = 32$ mm $s^{-1}$ (264). This value is more than 1,000-fold higher than the gas permeability coefficient of a $C_{16}$ lipid monolayer, about 20 μm $s^{-1}$ (13). The filling time for the larger *Anabaena* gas vesicle would be $t_e = 0.55$ μs, assuming the same value of κ.

In the gas vesicle suspension the gas vesicles would have been randomly oriented. If they had been oriented parallel to the diffusion gradient, however, it is calculated that they would have provided a channel with a diffusivity about threefold higher than in water (264). Oriented bundles of gas vesicles might therefore increase gas permeabilities in cells, but whether this would provide any benefit is another question. I doubt it; the exponential folding time for gas diffusion across the 5-μm diameter of a cell would be only 3.1 ms. Across a 1-mm colony of such cells the folding time for diffusion is much greater, 125 s, but gas vesicles occupy too small a proportion of the volume of a colony to have much effect on diffusion rates.

**Pores in the gas vesicle wall.** Gas molecules permeating the gas vesicle must pass through pores, large enough to accommodate them, that are located either between or within the gas vesicle proteins. From measurements of the gas permeability coefficient, κ, it is possible to determine the number of pores of a given size (or, vice versa, the size of the pores if their number were known) by using the kinetic theory of gases. In summary, this is done by calculating the average time it would take before a gas molecule of known collision diameter, bouncing around inside a gas vesicle at the speed of sound, finds a trajectory that leads out of a single pore in the wall (245).

The pores in the gas vesicle wall probably occur alongside other periodic structures. If there were one pore in each of the 107 ribs of the average *Anabaena* gas vesicle, for example, such pores would have a time-averaged diameter of 1.2 nm, about the size of one of the GvpA molecules; if there were a pore associated with each of the 21,000 GvpA molecules, however, they would need a diameter of only 0.44 nm to account for the observed value of κ (264). It is noted that the molecular model of the gas vesicle structure contains an opening of this size adjacent to the β-turn of each GvpA (Fig. 11).

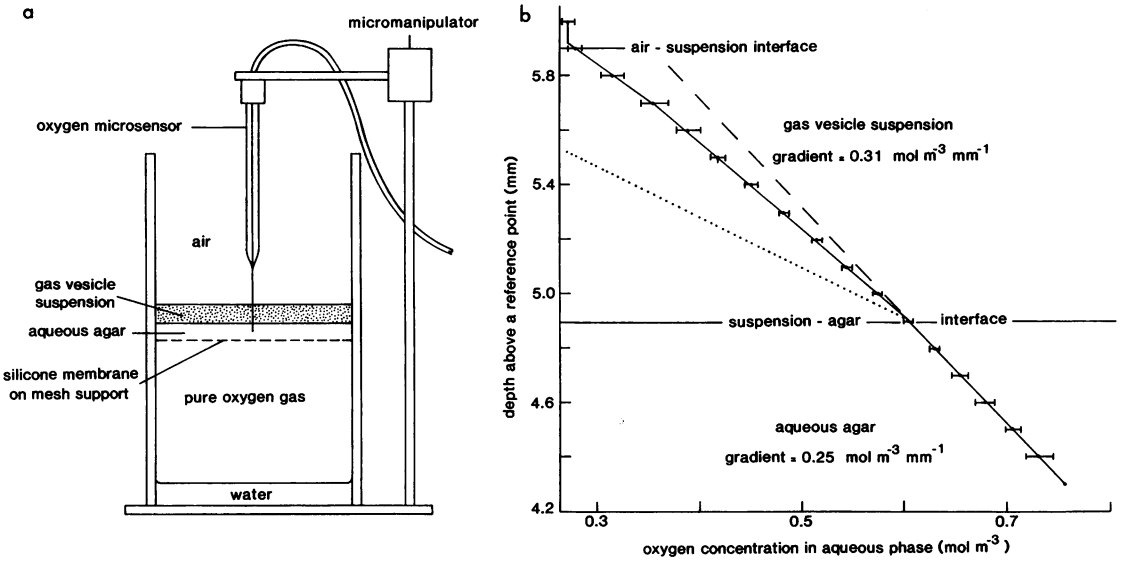


FIG. 18. (a) Apparatus used to determine the diffusion coefficient of oxygen through a 1-mm layer of gas vesicle suspension over a 1-mm layer of agar. (b) Gradients of oxygen concentration through the gas vesicle and agar layers, determined with the oxygen microsensor after a 12-h incubation. The diffusivity of oxygen through the gas vesicles can be calculated from the ratio of the gradients. The dashed line is the extrapolated gradient in agar, and the dotted line is the gradient expected for a suspension of impermeable particles occupying the same volume fraction as the gas vesicles. Redrawn from Walsby et al. (264) with permission from the publisher.

This kinetic analysis provides an explanation for the short equilibration times across the gas vesicle wall. The great rapidity with which the gas vesicle fills is a consequence of its minute size, as can be illustrated by returning to our million-fold larger flowerpot analogy. First, if the walls of the pot were to be formed with 2-nm layers of gas vesicle protein, the exponential filling time for the pot would be a million times longer than for the gas vesicle (because $t_e = 1/\alpha = V/A\kappa$). Second, if the walls were scaled up, from a thickness of 2 nm to 2 mm, the permeability coefficient of the wall would decrease a further millionfold. The exponential filling time of the macroscopic pot would therefore be $10^{12}$ times longer, about 5 days instead of 0.4 μs.

## Mechanics of the Gas Vesicle

The gas vesicle is a rigid structure that undergoes little decrease in volume when subjected to low pressures but collapses irreversibly at higher pressures. All of the essential properties (providing a gas-filled space, buoyancy, and light scattering) are lost when it collapses. The "critical" pressure at which collapse occurs varies widely in different organisms. The reasons for this have emerged from studies of the mechanical properties of the structure.

**Rigidity and elastic compressibility.** Klebahn (114) observed gas vesicles in a special chamber under the light microscope and could detect no change in their size when he applied pressure to them; he argued that they must therefore be different from gas bubbles and proposed that they must be rigid structures. They are rigid, but his evidence for this is inadmissable. A bubble of 1 μm in diameter in a cell would have an internal gas pressure of 0.8 MPa (0.1 MPa from atmospheric pressure, 0.3 MPa from surface tension, and 0.4 MPa from cell turgor) (251). Doubling the external pressure to 0.2 MPa would raise the internal pressure to 0.9 MPa, which would cause its volume to decrease by (1 − 8/9) and result in a change in diameter of only $[1-(8/9)^{1/3}] = 4\%$ (to 0.96 μm); this change would be undetectable. For similar reasons the observation that the flotation velocity of gas-vacuolate cyanobacteria is unchanged by application of a vacuum (228) provides no reliable indication of rigidity.

I have demonstrated the rigidity of the gas vesicle by measuring the compressibility of a concentrated suspension of isolated gas vesicles in a glass vessel, somewhat resembling a thermometer, with a fine-bore capillary attached to a bulb that could be filled and closed with a tight-fitting stopper. This "compression tube" (Fig. 19), filled nearly to the end of its open-ended capillary, was placed in a stout glass pressure tube in which the gas pressure could be raised (244, 260). On application of a moderate pressure ($P$), the gas vesicle suspension contracted and its change in volume ($\Delta V$) could be calculated from the movement of the meniscus in the capillary (after allowing for volume changes of the water and the glass tube). When the pressure was released, the meniscus returned to its original position, indicating that no gas vesicles had been collapsed. When a higher pressure (1.3 MPa) was applied, all of the gas vesicles collapsed; when the pressure was released, the meniscus returned to a new position. The volume between the two positions in the tube was equal to the volume of gas in the gas vesicles collapsed, $V_g$.

Gas vesicles isolated from *Anabaena flos-aquae* showed a reversible linear compressibility ($\Delta V/V_g$) of 0.0155 $MPa^{-1}$; i.e., their volume shrank by only 1 part in 645 when a pressure of 1 bar was applied (244). The gas vesicles of *Microcystis* sp. are narrower (cylinders of radius $r$ = 31.6 nm) than those of *Anabaena flos-aquae* (radius, 42 nm), and consequently they have both a lower compressibility (252) and a greater strength (252, 254). This can be explained by analyzing the mechanics of the gas vesicle.

When a pressure ($p$) is applied to a closed hollow cylinder such as the gas vesicle, it generates on the cross-sectional area ($A$) of the structure a force ($F$) that is equal to the pressure multiplied by the area ($F = pA$). This force is transmitted to the cross-sectional area of the wall ($A_w$), in which it produces a compressional stress ($-s$) equal to the force divided by the

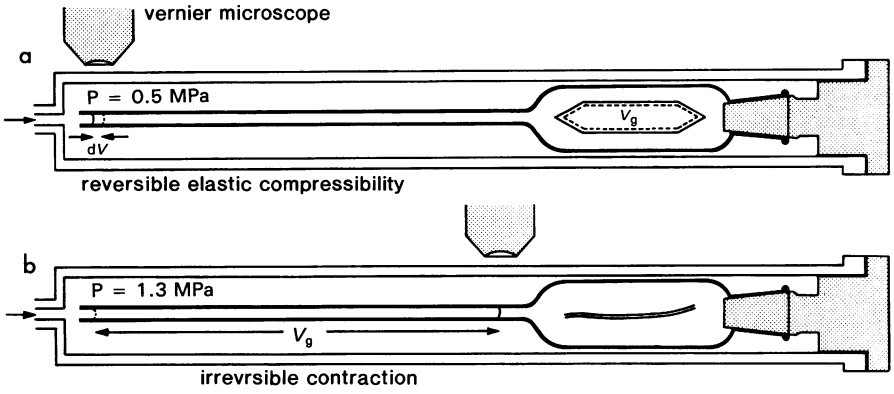


FIG. 19. Principle of the compression tube used in making measurements of gas vesicle compressibility and gas volume. The inner compression tube is filled with the gas vesicle suspension into the 0.1-mm precision bore capillary. The position of the meniscus in the capillary is located with a vernier microscope. When the gas pressure in the outer pressure tube is raised by a small amount, the pressure is transmitted to the suspension in the open capillary. Volume changes are indicated by the change in position of the meniscus: (a) reversible changes due to elastic compressibility of the gas vesicle; (b) irreversible changes due to collapse of the gas vesicle (the gas dissolves). Modified from Walsby (244) with permission from the publisher.

wall area ($-s = F/A_w$); extensional stresses are conventionally regarded as positive, and compressional stresses are negative. Hence $-s = pA/A_w$. Two important consequences emerge.

The first is that the stress in the wall of a compressed cylinder is proportional to $pr/t$, where $r$ is the cylinder radius and $t$ is the wall thickness. The gas vesicle wall is likely to be of a similar thickness ($t$ = 1.8 nm) in different species because it is made up of homologous proteins. The stress will therefore be proportional to the radius; i.e., wider gas vesicles will have a higher wall stress and a higher compressibility.

The second consequence is that because the ratio of $A/A_w$ varies in different directions, the stress varies in different directions within the wall. For geometrical reasons, the longitudinal compressional stress ($-s_l$) in the wall is

$$-s_l = p\pi r^2/2\pi rt = pr/2t \quad (4)$$

whereas the same pressure generates a transverse stress ($-s_t$) in the wall of

$$-s_t = p2rl/2tl = pr/t \quad (5)$$

Hence the transverse stress is twice the longitudinal stress. If a cylindrical engineering structure is to be reinforced with rods or fibers to withstand stresses, it is necessary to put twice as many running transversely around the cylinder as longitudinally along it. Alternatively, fibers may be wound diagonally at such an angle that the resultant stresses set up in them from the two directions are equal; the unique angle to the long axis of the cylinder at which this happens, sometimes referred to as the magic angle by engineers, is

$$\phi = \tan^{-1}\sqrt{2} = 54.7° \quad (6)$$

The reader may be familiar with hosepipes reinforced by nylon cord, which is ideally oriented at this angle. It has been noted (248) that the hydrogen bonds between the β-chains of GvpA of the gas vesicle are oriented within 1° of the magic angle (i.e., at 53.8° to the principal axis of the cylindrical gas vesicle). In such an orientation they will be subjected to the least distortion.

The stresses in the gas vesicle wall determine how much the structure is compressed and, ultimately, the pressure at which it collapses. These two properties are now analyzed in further detail (244, 252).

**Young's modulus of the gas vesicle wall.** The compressibility of the gas vesicle is determined by two elastic properties of the protein that constitutes its wall, Young's modulus and the Poisson ratio, which I will briefly explain.

Imagine a rod of length $L$ and cross-sectional area $a$ made of gas vesicle protein. A small compressional force applied to the ends of the rod induces in it a stress, $-s$, so that it changes length by an amount $-\Delta L$. The relative length change $-\Delta L/L$ is the strain, and the ratio of the stress to the strain gives a measure of the stiffness of the material, known as Young's modulus ($Y$):

$$Y = -s/(-\Delta L/L). \quad (7)$$

As the force is applied, not only does the rod get shorter but also its width, $w$, increases by an amount $\Delta w$. The ratio of this lateral strain ($\Delta w/w$) to the longitudinal strain ($-\Delta L/L$) is known as the Poisson ratio ($\sigma$). We do not know its value for the gas vesicle protein, but an average value for Newtonian solids is 0.33.

We now recast the protein in the rod into the shape of a gas vesicle, with a cylinder of radius $r$ and wall thickness $t$. We subject this gas vesicle to a pressure $p$ and measure how much it shrinks in volume. (Note that when we do this, the pressure of the gas remains at 1 bar because the gas remains in equilibrium with the air dissolved in the surrounding medium, whose concentration is not affected by the hydrostatic pressure per se.) The cylinder becomes narrower and shorter to a degree determined by its relative dimensions and by $Y$ and $\sigma$. The overall relative volume change can be calculated (157) to be

$$\Delta V/V = pr(4\sigma - 5)/2Yt \quad (8)$$

This equation can be used to determine Young's modulus of the gas vesicle from measurements of its cylinder radius, wall thickness, and compressibility. (Although $\sigma$ is not precisely known, the likely range of values gives only a 20% variation in $Y$.) For the *Microcystis* gas vesicle protein, $Y$ = 3.8 GPa (252). A somewhat lower value, 2.8 GPa, was found for the *Anabaena* gas vesicle protein (244); this may be explained by the deteri-

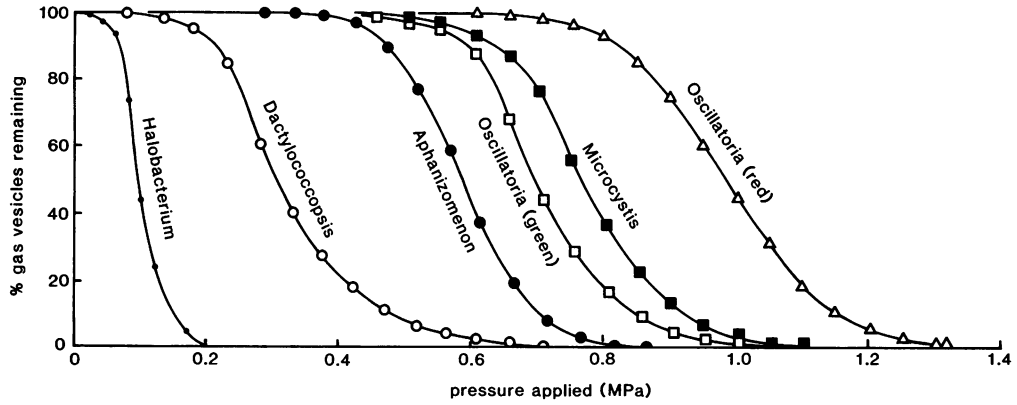


FIG. 20. Critical pressure distributions of gas vesicles from various species of cyanobacteria and a halobacterium (230). Modified from Walsby and Bleything (254) with permission from the publisher.

oration of these gas vesicles on storing, with loss of the outer layer of GvpC. A comparison with the stiffness of other substances is made below.

The low compressibility of the gas vesicle is important in natural habitats. The hydrostatic pressure in a water column rises by 0.01 MPa/m of depth. Ignoring turgor pressure, if a *Microcystis* cell were to be carried down to a depth of 10 m, its gas vesicles, which contract by only 0.9% per MPa, would shrink by less than 0.1%. They would not, therefore, lose their effectiveness as buoyancy aids (as long as they were not collapsed by the pressure).

**Mode of gas vesicle collapse.** Gas vesicles collapse when exposed to a sufficient hydrostatic pressure. The central cylinder collapses to a flat rectangular envelope; the conical end caps collapse to sectors of circles that, because of their curved edges, form a tangential contact with the ends of rectangle. As the cylinder collapses, its cross-section must deform throughout its length and splits must propagate parallel to one another on diametrically opposite sides along the entire length, to enable the collapsed structure to lie flat. Evidence that the structure does lie flat is provided by its tendency to form scrolls or to fold (230). In *Microcystis* gas vesicles the folds occur at an angle of 60 ± 8° (108); perhaps they fold along the β-chains that lie at an angle of 54° to the principal axis of the gas vesicle. The regularity of the collapsed structures must stem from the crystalline perfection of the wall.

Engineers describe two modes of collapse in hollow, cylindrical structures (44). In the first mode, typical of thick-walled structures of low strength, collapse occurs when the transverse stress reaches the yield stress, $s_y$, of the material. The yield pressure, $p_y$, of the cylinder that collapses without previous distortion is then given by

$$p_y = -s_y t/r \quad (9)$$

This equation applies both when excess pressure is applied on the outside of the structure ($s$ negative), causing collapse, and when it is applied on the inside ($p$ negative, $s$ positive), causing explosion. Thin-walled cylinders constructed of high-strength materials collapse by the second mode, instability failure. At a pressure below $p_y$ the cylinder goes out of round and buckles, so that the forces become concentrated in the more highly curved regions. A "runaway" situation develops, and the structure collapses when the yield stress is reached at this highly curved edge. Such instability does not occur when the excess pressure is on the inside, because the wall is uniformly under tension and no buckling results. This may, perhaps, be more easily understood by returning to the analogy of our rod of gas vesicle protein. Under tension the rod keeps straight and remains intact until the extensional yield stress ($s_y$) is reached. Under compression the rod bends and then snaps at a much lower load.

The flattened form of the collapsed gas vesicle clearly indicates instability failure, and this is confirmed by the finding that the critical collapse pressure is much lower than the pressure at which gas vesicles explode (252).

**Critical pressure.** The critical pressure (or critical collapse pressure) is the minimum difference in pressure between the outside and the inside of the gas vesicle that causes the structure to collapse (230). The only source of pressure on the inside is the gas, which is always in equilibrium with the gas dissolved in the suspending medium. The gas vesicle gas is therefore usually air at the ambient pressure and balances the overlying air pressure. Consequently, the critical pressure ($p_c$) can be functionally defined as the gauge pressure at which a gas vesicle collapses in a suspension (previously) equilibrated with air. Measurement of critical pressures by nephelometry is discussed below.

The mean critical pressures of gas vesicles vary over a wide range, from less than 0.1 MPa (1 bar) in halobacteria (230) to more than 3.5 MPa (35 bars) in the oceanic cyanobacterium *Trichodesmium thiebautii* (237). Within each species the critical pressure of individual gas vesicles may vary two- to threefold, for example between 0.75 and 1.25 MPa in *Oscillatoria agardhii* and 0.2 and 0.6 MPa in *Dactylococcopsis salina* (254). There may be a similar range of critical pressures for the gas vesicles within a single cell (246) (see Fig. 24). These critical pressure distributions or collapse pressure curves have been investigated quantitatively by measuring either the turbidity change (230, 232) (see Measurement of Light Scattering by Gas-Vacuolate Cell Suspensions, below) or the volume change (244, 260) (Fig. 20) that accompanies the gas vesicle collapse.

Part of the variation in critical pressure in different species can be explained by the variation in cylinder radius of the gas vesicles. From the theory of mechanics (see Buckling Pressure, below), it is expected that the collapse pressure will vary inversely as some function of radius (equation 9) (Fig. 21). Surveys of gas vesicles in eight genera of cyanobacteria (86, 254) in which the mean cylinder radius varied from 31 nm in an *Oscillatoria* strain to 55 nm in *Dactylococcopsis salina* showed (empirically) that the mean critical pressure was inversely correlated with radius according to the relationship

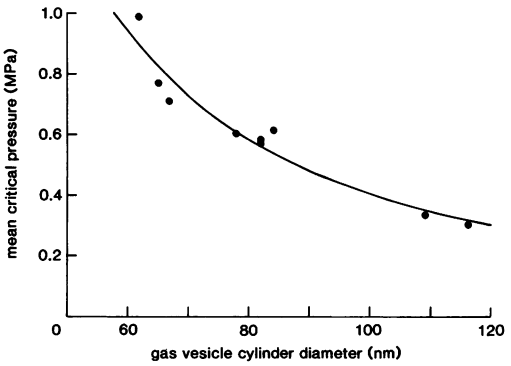


FIG. 21. Relationship between critical pressure ($p_c$) and cylinder radius ($r$) of gas vesicles in different cyanobacteria. Reprinted from Walsby and Bleything (254) with permission from the publisher. Additional data are from R. Kinsman.

$$p_c = 275\ (r/\text{nm})^{-1.67}\ \text{MPa} \approx 275\ (r/\text{nm})^{-5/3}\ \text{MPa} \quad (10)$$

The same general trend holds when extrapolated in either direction: the narrowest gas vesicles recorded ($r$ = 23 nm) occur in *Trichodesmium thiebautii* (69), in which the highest critical pressures have been measured ($p_c$ = 3.7 MPa [237]); the widest occur in halobacteria, in which $r$ can exceed 100 nm and $p_c$ = 0.09 MPa (230).

Part of the variation in critical pressure within each species can also be attributed to variation in cylinder width. In *Anabaena flos-aquae* the variation in cylinder radius accounts for about half of the critical pressure variation (254).

No similar correlation has been found between the critical pressure and the length of the gas vesicles. It has been concluded that the individual ribs derive little support from the ends of the structure via their neighbors (230).

**Buckling pressure.** For an unstiffened cylinder, there is a theory that relates the buckling pressure to Young's modulus of the wall material (4). When the axial ratio of the cylinder is between 2 and 10 (252), the expected buckling pressure is given by

$$p_b = Yt^3/4r^3(1 - \sigma^2) \quad (11)$$

Substituting the values used previously, $Y$ = 3.8 GPa, $t$ = 1.8 nm, $\sigma$ = 0.33, and $r$ = 31.2 nm, the *Microcystis* gas vesicle would have a predicted buckling pressure of 0.18 MPa (252). This value is considerably lower than the mean critical pressure of the intact gas vesicle (about 0.8 MPa) but quite close to the mean critical pressure (0.23 MPa) of gas vesicles that have had the outer protein, GvpC, removed (258). It can be envisaged that each rib has a weak spot in a different position and therefore, when under pressure, tends to go out of round at a different position. Such distortion is resisted by the straps of GvpC molecules, which bind the ribs together and thereby provide a sort of corset that prevents the gas vesicle from bulging out.

The advantages of providing stiffening to postpone buckling should increase with cylinder width. By using the coefficients $Y$, $t$, and $\sigma$, we can rewrite equation 11 in the same form as equation 10:

$$p_b = [Yt^3/4(1 - \sigma^2)]r^{-3} = 6{,}217(r/\text{nm})^{-3}\ \text{MPa} \quad (12)$$

Then by combining equations 10 and 12, we obtain

$$p_c/p_b = 0.0442(r/\text{nm})^{4/3} \quad (13)$$

and from this it follows that the ratio of $p_c/p_b$ increases with $r^{4/3}$ (Fig. 21). Furthermore, if the difference between $p_c$ and $p_b$ is caused by the stiffening provided by the outer gas vesicle protein, this will provide a greater improvement for wide gas vesicles, such as those found in halobacteria and halophilic cyanobacteria.

The quantitative value of stiffening the structure with GvpC can be calculated by comparing the amount of protein required to make stiffened and unstiffened gas vesicles that would withstand a particular pressure. Consider the *Anabaena* gas vesicle with a critical pressure of 0.56 MPa and a radius of 42 nm: for one of average length (470 nm), the ratio of gas space ($V_i$) to wall volume ($V_w$) is 10.1:1 (see Fig. 25 and the equations of Walsby and Bleything [254]). GvpC, which provides the stiffening of this structure, accounts for about 10% of the wall volume (22). According to equation 12, an unstiffened cylinder with a buckling pressure of 0.56 MPa would have a radius of 22 nm; a 470-nm-long gas vesicle of this radius would have a $V_i/V_w$ ratio of 5.1:1 (see Fig. 25). Providing the same volume of gas space with an unstiffened gas vesicle would therefore require the investment of nearly twice the amount of wall protein.

**Yield stress: exploding gas vesicles.** It has been explained that the gas pressure inside a gas vesicle can be raised by infiltration with gas from an overlying gas phase and that gas vesicles can be equilibrated under high gas pressures (230). If the overlying gas pressure is then lowered, the gas pressure inside the gas vesicle, $p_i$, will exceed the pressure on the outside, $p_o$, while the suspension outgasses. If the overlying gas pressure is lowered rapidly enough, the pressure difference between the inside and outside may be sufficient to cause the gas vesicle to explode (252). No buckling occurs with excess pressure on the inside (just as no buckling occurs with a rod under tension); consequently, failure of the gas vesicle will not occur until the yield pressure is reached, i.e., until

$$p_o - p_i = -p_y = s_y t/r \quad (14)$$

The yield pressure has been determined so far only with the gas vesicles of *Microcystis* sp., which have a mean $p_y$ of 4.3 MPa (43 bars). This is five times greater than the mean critical pressure of these gas vesicles. It confirms that collapse under external pressure occurs through instability failure due to buckling, even though this is postponed (also approximately fivefold) through the stiffening provided by GvpC. By rearranging equation 9, the yield stress of the *Microcystis* gas vesicle wall can be determined: $s_y$ = 4.3 MPa × 32.6 nm/1.8 nm = 78 MPa (252).

The Hemmingsens (88) performed experiments in which gas-vacuolate cells of *Ancylobacter* (*Microcyclus*) *aquaticus* were infiltrated with gas and then rapidly decompressed. The cells were killed after decompression from 10 MPa (but not 5 MPa), presumably because they were damaged by bubbles of gas that expanded from the gas vesicles. A quantitative analysis of the amount of gas present supports the conclusion that the gas vesicles would have exploded and formed bubbles within the cells (251).

**Mechanical properties at the molecular level.** In making the flowerpot analogy, I commented that some of the physical properties encounter problems when modeled at different sizes. The mechanical properties do not suffer in this way as long as the model is "constructed" to scale throughout. Nevertheless, it is rather fascinating to see the theory of mechanics apply to a structure that has a wall that is only one molecule thick (or two where GvpC crosses).

A check on the validity of these analyses has been provided by comparing two properties of the protein that makes the gas

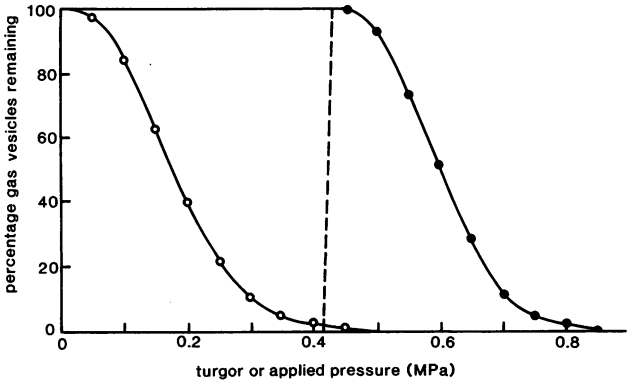


FIG. 22. The critical collapse pressure of gas vesicles isolated from *Anabaena flos-aquae* varies from about 0.45 to 0.85 MPa (solid symbols). Gas vesicles inside the turgid cells are collapsed by application of 0.05 to 0.45 MPa (open symbols) because they are already subjected to a cell turgor pressure of about 0.4 MPa. The difference between the two curves gives a measure of the turgor pressure. The turgor pressure falls slightly as the gas vesicles are collapsed (see Fig. 23 for an explanation). Redrawn from Walsby (239) with permission from the publisher.

vesicle wall with those of nylon (252). This is a polymer of -[CO-NH-$(CH_2)_n$]- forming β-chains which are hydrogen bonded in the same way as polypeptide β-sheet (Fig. 11). Young's modulus of the densest nylon is 4 GPa, and the yield stress of this material is 83 MPa (8); these values are close to those of the gas vesicle protein, 3.8 GPa for Young's modulus and 78 MPa for the yield stress (252). In mechanical terms, a coil pot made of nylon ribs, suitably stiffened with fibers, would refine our model of the gas vesicle.

It is sometimes claimed that through continual refinement by natural selection, nature comes up with the best solutions. This is true only within the constraints of the rather limited range of materials it has available. Using titanium, a hollow cylinder could be constructed that was stiffer, stronger, and lighter than the gas vesicle: forging structures from metal, however, is not within the repertoire of organic evolution.

**Gas vesicle collapse in cells: turgor pressure.** The collapse of gas vesicles inside cells is governed by the same principles that apply to isolated gas vesicles, but there are several factors in cells (such as temperature, pH, salinity, surfactants, and enzymes) that may affect the stability of the gas vesicle protein and hence the critical pressure of the structures (23). The most important factor, however, is cell turgor pressure (Fig. 22) (230).

The cells of bacteria are usually distended by a turgor pressure ($p_t$) that equalizes the difference in water potential between the outside and inside of the cell. This turgor presses out on the cell wall and in on the gas vesicle and contributes to pressures causing collapse of the structures. The first measurement of turgor pressure per se (i.e., rather than solute potential) in bacteria was made by using gas vesicles as pressure sensors (230). The mean critical pressure ($p_c$) of gas vesicles was determined in cells suspended in hypertonic sucrose solution, which removed the turgor pressure; the mean apparent critical pressure ($p_a$) of the gas vesicles was measured in another sample of the turgid cells, suspended in the dilute culture medium (Fig. 22); the turgor pressure could then be calculated from the difference in these two measurements:

$$p_t = p_c - p_a \quad (15)$$

This simple measurement gives a result that, for cyanobacteria, is usually within a few percent of the original turgor pressure. There are a number of refinements that may be needed to give more precise values in these (239) and other (123, 234) organisms, the most important of which concerns the loss of turgor pressure as gas vesicles are collapsed (Fig. 23).

For this review the principal interest of turgor pressure is its role in the regulation of gas vesicles themselves; in some organisms, gas vesicles can be collapsed when the turgor pressure rises (110, 162). Cell turgor pressure is not static but increases with the concentration of ions or other small molecules in the cytoplasm. Measurements by the gas vesicle method have shown that turgor pressure rises in cyanobacteria exposed to high photon irradiances (46, 230); about half of the rise in turgor can be attributed to an increase in the concentration of low-molecular-weight photosynthetic products (77),

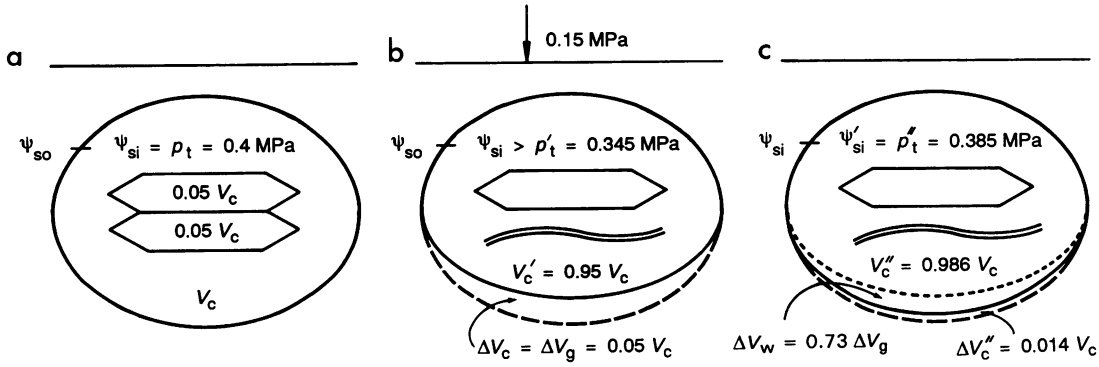


FIG. 23. Pressure-volume relations of a gas-vacuolate cell: the loss of turgor pressure as gas vesicles collapse. (a) An *Anabaena* cell with a turgor pressure $p_t$ = 0.4 MPa, equal to the difference in solute potential outside ($\psi_{so}$) and inside ($\psi_{si}$) the cell. The turgor pressure presses out on the cell wall, stretching it to give a cell volume $V_c$; it also presses in on the gas vesicles of volume $V_g$ = 0.1 $V_c$ and mean critical pressure 0.55 MPa. The cell has a volumetric elastic modulus of $\varepsilon$ = 1.1 MPa. (b) A pressure ($p_a$) of 0.15 MPa is suddenly applied outside the cell; instantaneously the pressure in the cell rises to $p_t + p_a$ = 0.55 MPa; half of the gas vesicles (volume $\Delta V_g$) collapse, and the cell volume decreases by $\Delta V_c = 0.05V_c$. The turgor pressure therefore decreases by $\Delta p = \varepsilon \Delta V_c/V_c$ = 0.055 MPa to a new value of $p_t$ = 0.345 MPa. The turgor pressure is now lower than the difference in solute potential. (c) Water therefore enters, raising the turgor but diluting the cell sap, until a new equilibrium is reached. The volume of water that enters, $\Delta V_w$, is approximately $0.73\Delta V_g = 0.036V_c$ (239, 260); hence the overall volume change is $0.014V_c$; the overall turgor change is $\Delta p_t = 0.014\varepsilon$ = 0.015 MPa, and the final turgor pressure is 0.385 MPa.

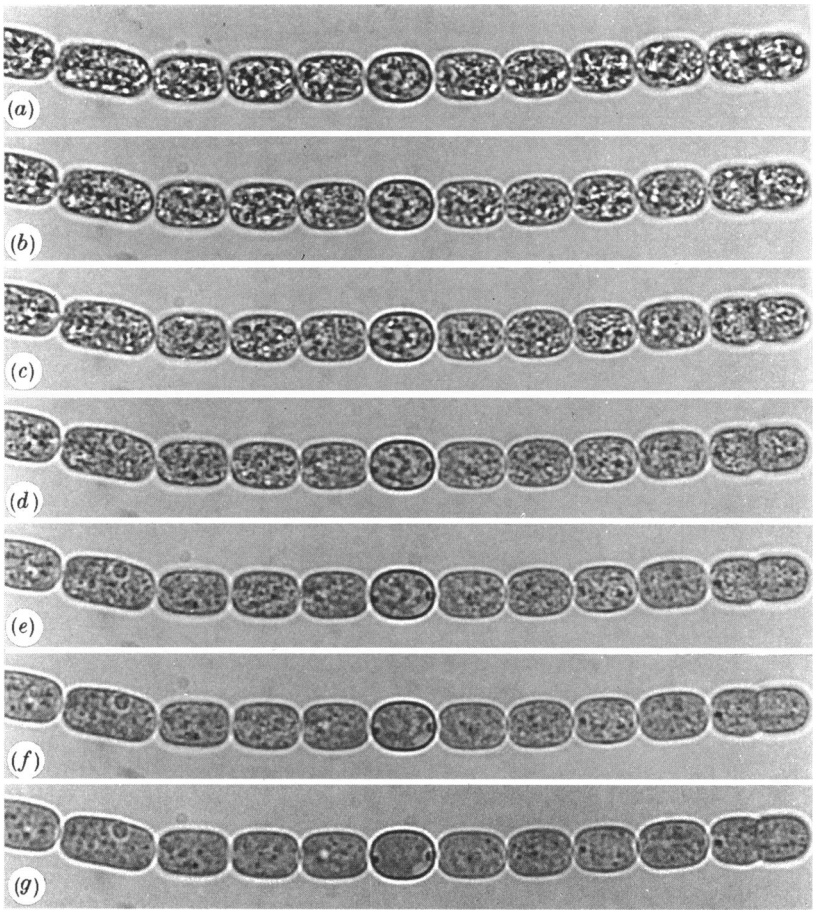


FIG. 24. Cells of the filamentous cyanobacterium *Anabaena flos-aquae* each contain as many as 5,000 gas vesicles in aggregates—gas vacuoles—which are visible under the light microscope (a). The cells have a turgor pressure of about 0.38 MPa; the individual gas vesicles, whose critical collapse pressures range mostly from 0.50 to 0.75 MPa, with a mean of 0.60 MPa, collapse when sufficient additional pressures are applied. They collapse independently of one another, and the gas vacuoles become less distinct. Serial photographs of a filament after application of pressures of (a) 0 MPa above atmospheric pressure (no gas vesicle collapse), (b) 0.15 MPa (causing 21% gas vesicle collapse), (c) 0.2 MPa (41% collapse), (d) 0.25 MPa (66% collapse), (e) 0.30 MPa (82% collapse), (f) 0.35 MPa (92% collapse), (g) 0.70 MPa (100% collapse). Magnification, ×1,260. Note that the collapse sequences in the dividing cell (right) and the fully grown cell (left) are similar to those in the other cells; from this it is concluded that the turgor pressure of the cells changes little during the cycle of cell growth and division. The central cell with a heavier envelope is an immature heterocyst. Reproduced from Walsby (246) with permission from the publisher.

and about half can be attributed to the light-stimulated uptake of potassium salts (5). Such light-dependent rises in turgor may be an inevitable consequence of increased photosynthetic activity and perhaps occur in all cyanobacteria. They have now been demonstrated in a number of gas-vacuolate species of *Anabaena* (46, 110, 230, 266), *Aphanizomenon* (128, 129, 137), *Gloeotrichia* (252a), *Nostoc* (7), *Oscillatoria* (125, 215, 262), and *Microcystis* (138, 184, 212). Gas vesicle regulation by cell turgor is discussed below (see Gas Vesicle Collapse by Turgor Pressure).

The measurement of turgor pressure is also relevant to a number of other fields such as cell growth and extension of the cell wall under stress. Pinette and Koch (177) have made observations on the pressures at which gas vesicles started to collapse in cells of *Ancylobacter* (*Microcyclus*) *aquaticus* viewed individually under the microscope; from such measurements it is possible to infer the turgor pressure in the individual cell, as long as the range of critical pressures of gas vesicles is similar in all cells. Koch (122) has commented that the turgor pressure may remain nearly constant during cell growth and division (under given conditions). Support for this idea can be found in observations made on gas vesicle collapse in the chains of cells in filaments of cyanobacteria (Fig. 24).

Turgor pressure has also been implicated in osmoregulation by bacteria which, stressed by a decrease in the external solute potential, adjust their internal solute potential by active uptake of potassium or by synthesis of compatible solutes. It is postulated that the recovery of turgor pressure might be controlled by turgor sensors; such changes in turgor pressure have been demonstrated in gas-vacuolate cyanobacteria (183). The gas vesicle method of turgor pressure measurement has also provided a means of measuring the permeability to solutes, the hydraulic conductivity ($L_p$), and the volumetric elastic modulus ($\varepsilon$) of cells (239) (Table 2). In the cyanobacteria *Anabaena flos-aquae* (239) and *Microcystis* sp. (183), there is a linear change in relative cell volume ($\Delta V/V_c$) with increases in turgor pressure ($\Delta p_t$), as predicted by the mechanics of a pressure vessel (157), i.e.,

$$\varepsilon = \Delta p_t V_c / \Delta V \quad (16)$$

TABLE 2. Measurements of mean gas vesicle critical pressure ($p_c$), cell turgor pressure ($p_t$), volumetric elastic modulus ($\epsilon$), and hydraulic conductivity ($L_p$) by the gas vesicle method

| Organism | $p_c$ (MPa) | $p_t$ (MPa) | $\varepsilon$ (MPa) | $L_p$ ($\mu m\ s^{-1}\ MPa^{-1}$) | Reference(s) |
|---|---|---|---|---|---|
| Cyanobacteria | | | | | |
| *Anabaena flos-aquae* | 0.60 | 0.43 | 1.1 | 1.4 | 239 |
| *Aphanizomenon flos-aquae* | 0.60 | 0.35 | | | 128, 254 |
| *Dactylococcopsis salina* | 0.33 | 0.08 | | | 254, 268 |
| *Microcystis* sp. | 0.76 | 0.32 | 1.3 | | 183 |
| *Nostoc muscorum* | 0.53 | 0.36 | | | 7 |
| *Oscillatoria agardhii* | 0.99 | 0.38 | | | 254, 267 |
| *Prochlorothrix hollandica* | 0.89 | 0.46 | | | 252a |
| Other bacteria | | | | | |
| *Amoebobacter roseus*[a] | 0.34 | 0.15 | | | 230 |
| *Amoebobacter purpureus* | 0.24 | 0.10 | | | 166 |
| *Ancylobacter aquaticus*[b] | 0.50 | 0.19 | | | 123 |
| *Pelodictyon phaeochlathratiforme* | 0.49 | 0.33 | | | 164 |
| *Prosthecomicrobium pneumaticum* | 0.54 | 0.30 | | | 234 |
| Halobacterium | | | | | |
| *Halobacterium salinarium* | 0.09 | 0.00 | | | 230 |

[a] Synonym for *Rhodothece conspicua* (230).
[b] Synonym for *Microcyclus aquaticus* (219).

Other analyses predict nonlinear expansion (123), but they appear to neglect the Poisson ratio of the cell wall material.

## Surface Properties of the Gas Vesicle

**Outer surface.** Gas vesicles have a hydrophilic outer surface. When an aqueous suspension of gas vesicles isolated from *Anabaena flos-aquae* was shaken with olive oil, the gas vesicles remained in the aqueous phase. Freeze-dried gas vesicles could be readily resuspended in water but not in olive oil (230). These partition experiments indicate that the gas vesicle has a wettable outer surface. Water spreads on a surface coated with dried gas vesicles (230). This indicates that the contact angle, $\theta$, must be less than 90°. It should be measured.

If the outer surface of the gas vesicle were hydrophobic rather than hydrophilic, the interfacial tension ($\gamma$) between water and the curved surface would generate forces on the structure that might cause its collapse. The forces would not be the same in all directions. Parallel to the long axis of the cylinder there is no curvature: the force would be zero, and there would be no effect on the longitudinal stress in the wall. Tangential to the curved surface, however, the force generates a pressure, $p_\gamma$, on the longitudinal section (of length $l$) given by

$$p_\gamma = -\cos\theta(2\gamma l/2rl) = -\cos\theta\ (\gamma/r) \quad (17)$$

This pressure would generate in the wall a transverse stress, $-s_\gamma$, that is independent of the radius:

$$-s_\gamma = p_\gamma r/t = -\cos\theta(\gamma/t) \quad (18)$$

For a cylinder with a completely unwettable surface ($\theta = 180°$, $\cos\theta = -1$) with the same radius as the *Anabaena* gas vesicle ($r = 42$ nm), the pressure generated by surface tension (of $\gamma = 0.075\ N\ m^{-1}$, the value between air and water) would be $p_\gamma = 1.8$ MPa, which is three times greater than the critical pressure of *Anabaena* gas vesicles. These calculations clearly demonstrate the importance of a small contact angle. In fact, it appears from equation 17 that contact angles of less than 90° would result in a negative pressure on the surface, generating a tension within the wall that would help to support the structure and, during its formation, would help to erect it.

**Inner surface.** Collapsed gas vesicles dried down on a flat surface form closely packed layers on which there is an alternating sequence of pairs of inner-facing surfaces and pairs of outer-facing surfaces (15, 16). It has been shown by neutron diffraction studies that when such layers are rehydrated (with $D_2O$), water enters only between alternate surfaces, i.e., between either the apposed inner or outer surfaces (278). It follows that only one of the two surfaces is hydrophilic. Since it is known from partition experiments that the outer surface is hydrophilic, the inner surface must be the hydrophobic one. The contact angle, $\theta$, between water and the inner surface must therefore exceed 90°, but, again, its actual value is unknown.

The hydrophobicity of the inner surface prevents liquid water from entering the gas vesicle (207, 228). To drive liquid water through a pore of radius $r_b$ opening into a hydrophobic surface would require a pressure of $p_w = -\cos\theta(2\gamma/r_b)$ to overcome the forces of surface tension. The pressure required to force liquid water inside a pore with the same radius as the gas vesicle (42 nm) with a contact angle of, say, 140° ($-\cos\theta = 0.77$) would be 1.4 MPa (14 bars). Any pores that exist through the wall must, of course, be much smaller than the cylinder radius (see Pores in the Gas Vesicle Wall, above), and the pressure required must therefore be much higher.

The only other way for liquid water to accumulate inside the gas vesicle would be for water vapor to condense inside. To do this, it would have to form a droplet. The equilibrium vapour pressure ($p_v$) over the convex surface of a water drop of radius $r_d$ is higher than that over a planar surface ($p_w$):

$$p_v/p_w = e\ \exp(2\gamma M/r_d\rho RT) \quad (19)$$

where $M$ is the molar mass ($0.018\ kg\ mol^{-1}$), $\rho$ is the density of water ($998\ kg\ m^{-3}$), $R$ is the gas constant ($8.3\ kg\ m^2\ s^{-2}\ K^{-1}\ mol^{-1}$) and $T$ is the temperature (293 K at 20°C). Hence, when the radius of the drop is small enough to fit in the gas vesicle, say $r_d = 5$ nm, from equation, $19\ p_v/p_w = 1.25$; i.e., the vapor pressure would be 25% higher over the drop than over a planar water surface. By a similar argument, the vapor pressure in equilibrium with the concave water surface outside the gas vesicle, of $r = 42$ nm, would be 3% lower than that over a planar surface. The vapor would therefore condense outside

the gas vesicle and the drop inside would evaporate; conversely, liquid water could not form by condensation inside the gas vesicle.

When a gas vesicle first forms, the constituent proteins are presumably surrounded by water on all sides. At some point in the assembly of the gas vesicle, continuity must be lost between the liquid water on the outside and that which might remain inside the structure. As the enclosed space enlarges, that remnant of water would form a drop and then evaporate.

### Buoyant Density of the Gas Vesicle

The principal function of the gas vesicle is to provide cells with buoyancy (231). The efficiency with which this function is provided depends on the buoyant density of the gas vesicle, which in different organisms ranges from about 60 to 210 kg $m^{-3}$ (compared with 998 kg $m^{-3}$ for water). The case of the *Anabaena* gas vesicle will be used to explain the relationship between shape, size, and density.

From geometrical measurements made on gas vesicles isolated from *Anabaena flos-aquae* (Fig. 13), it has been calculated (253) that the "average" gas vesicle has a volume of $V = 2.35 \times 10^6$ $nm^3$, of which the gas space occupies $V_i = 2.14 \times 10^6$ $nm^3$ and the wall occupies $V_w = 0.211 \times 10^6$ $nm^3$. The mass of the wall in this gas vesicle, $M_w = 2.79 \times 10^{-21}$ kg, is calculated from the product of $V_w$ and the density of the gas vesicle protein, $\rho_w = 1{,}320$ kg $m^{-3}$ (15); similarly, the mass of gas, $M_g = 0.02 \times 10^{-21}$ kg, is calculated from $V_i$ and the density of gas, assumed to be moist air at atmospheric pressure, $\rho_i = 1.2$ kg $m^{-3}$. The gas occupies 91% of the volume but contributes less than 1% of the mass. The overall mass of the gas vesicle is therefore $M = 2.81 \times 10^{-19}$ kg, and its overall density is $\rho = M/V = 120$ kg $m^{-3}$ (254).

Similar calculations have been made for gas vesicles isolated from eight species of cyanobacteria. The average density was found to vary from 100 kg $m^{-3}$ in *Dactylococcopsis salina* to 162 kg $m^{-3}$ in *Oscillatoria agardhii* (254). The widest gas vesicles, found in halobacteria (see Fig. 10a in reference 231), are estimated to have a density of about 60 kg $m^{-3}$, whereas the narrowest, in *Trichodesmium theibautii* (69), may have a density exceeding 210 kg $m^{-3}$, 3.5 times higher.

This variation in the efficiency with which a gas vesicle provides buoyancy is closely related to the ratio $V_i/V_w$. Geometrical analysis (254) shows that this ratio increases in almost direct proportion to the width of the gas vesicle (Fig. 25), so that for reasons of efficiency a gas vesicle should be as wide as possible. The critical pressure of a gas vesicle has been shown to decrease with width (equation 10) (86, 230, 254), however, and the requirement to withstand a certain minimum pressure sets an upper limit to the width in each organism (230).

There appear to be no such constraints on the length of a gas vesicle, because the critical pressure is independent of length (230). Why, then, are gas vesicles not made much longer? Some gas vesicles reach a length of over 1 μm, and yet the average length in eight species (from seven genera) of cyanobacteria we investigated was only 470 nm (254). Geometrical analysis here shows that for a gas vesicle of a given width the increase in the ratio $V_i/V_w$ approaches a plateau at a length equivalent to about five widths (Fig. 25); this length is exceeded in seven of eight cyanobacteria that have been investigated (254). There would be little advantage in their being longer.

**Packing fraction of gas vesicles.** Many gas vesicles may be required to provide a cell with buoyancy. In cyanobacteria there may be more than 10,000 per cell (109). Ultimately it is the overall density of the gas vacuole (i.e., of the gas vesicles together with the fluid-filled spaces between them) that determines whether sufficient buoyancy can be provided to make a cell float.

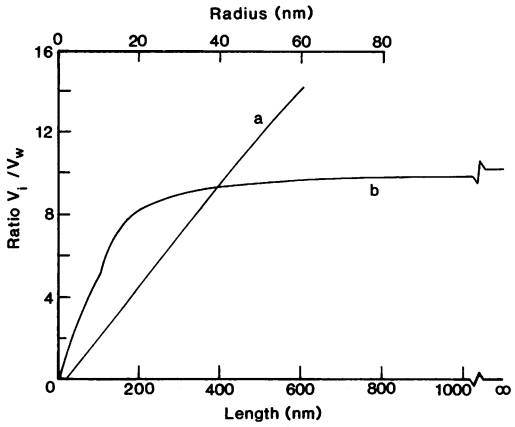


FIG. 25. Graph showing how the ratio of gas volume ($V_i$) to wall volume ($V_w$) changes with increasing radius in gas vesicles of average length (470 nm) (curve a) and with increasing length in gas vesicles of average radius (39.4 nm) (curve b); the kink occurs at the transition from the biconical form. Modified from Walsby and Bleything (254) with permission from the publisher.

Electron microscopy of thin-sectioned cells of several cyanobacteria (20, 107, 198, 272) and methanogens (279) has shown that cylindrical gas vesicles are usually closely stacked in hexagonal arrays, like the cells of a honeycomb. This arrangement is also demonstrated by freeze-fracturing (107). Geometrical analysis demonstrates that when cylinders of uniform radius are arranged in this manner, the proportion of the volume they occupy is $\pi/(2\sqrt{3}) = 0.907$. Gas vesicles are cylinders with conical ends. Adjacent layers of gas vesicles may be packed tightly together with the cones interdigitating. The maximum proportion of the volume that stacked cones can occupy is $\pi/[6(\sqrt{3} - 1)] = 0.715$. The overall proportion for the cylinders with conical ends will depend on the relative length of the cylinder ($l$) and height ($h$) of each of the two cones. For the *Anabaena* gas vesicle, in which $l = 7.4h$, the proportion would be 0.89. In practice the proportion must be somewhat lower because there is some variation in cylinder radius (254) (Fig. 1).

The *Anabaena* gas vesicle has a density of 120 kg $m^{-3}$. If the intervening space were filled with an aqueous solution of, say, density 1,000 kg $m^{-3}$ which occupied 15% of the volume, the overall density of the gas vacuole would be 252 kg $m^{-3}$, about a quarter of the density of water.

The cylindrical gas vesicles pack more closely than uniform spheres, which, in alternating hexagonal arrays, occupy only $\pi/(3\sqrt{2}) = 0.740$ of the available volume.

### Interactions of Gas Vesicles with Light

Gas vesicles, and the gas vacuoles into which they are aggregated, interact with light in a number of ways, which have been only partially described and explained. The remarkable optical properties of gas vesicles disappear when the structures are collapsed by pressure (Fig. 26); the changes in light scattering or attenuation can be used in measuring gas vesicles (230, 232) and investigating the nature of the optical phenomena (49, 158, 179, 225, 229). It has been suggested that gas vesicles may provide light shielding in cells (217, 225, 229), but in examples investigated it is either rather insignificant (192) or

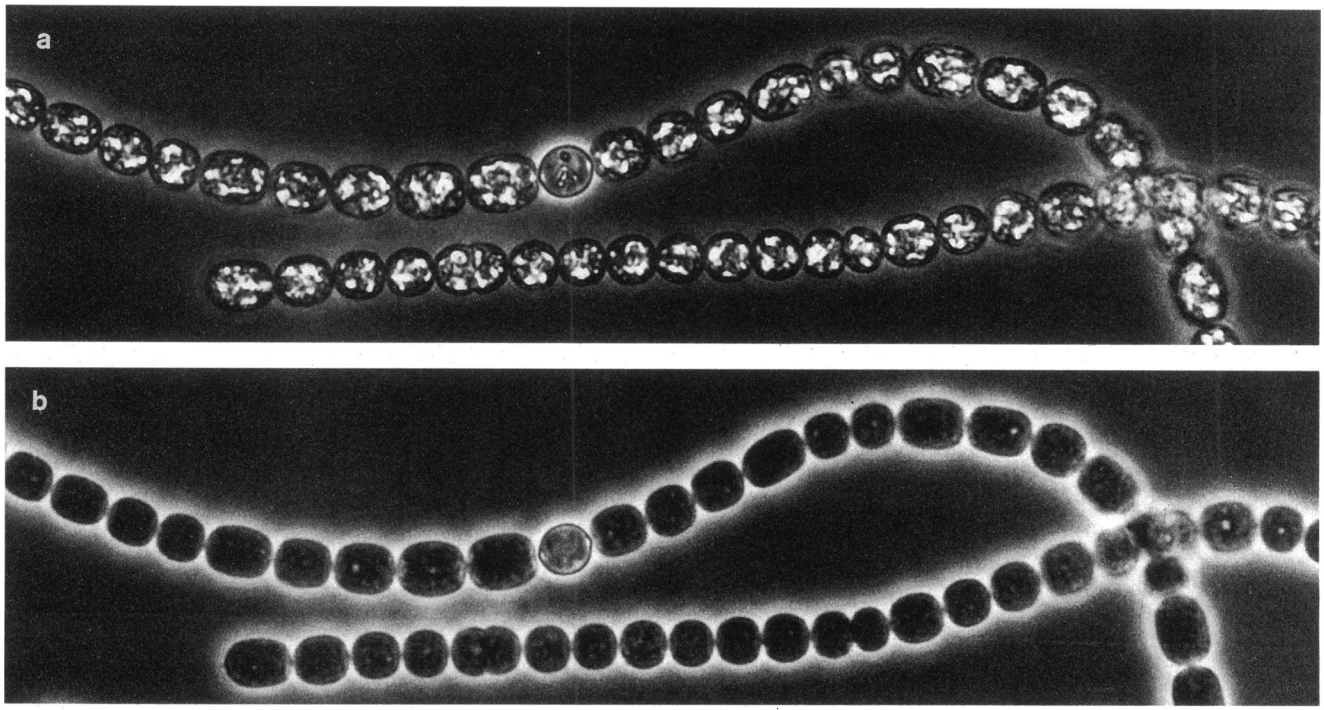


FIG. 26. Phase-contrast light micrographs of filaments of the cyanobacterium *Anabaena flos-aquae* before (a) and after (b) collapse of their gas vesicles by application of a pressure of 1.0 MPa (10 bar). The cells are approximately 5 μm wide.

quantitatively undefined (158). Gas vesicles do increase light attenuation in suspensions (179, 220) and natural populations (68), and this can affect light penetration in lakes.

**Light scattering by isolated gas vesicles.** Suspensions of isolated gas vesicles appear milky white. The intact gas vesicles do not absorb light in the visible spectrum, but they scatter light strongly (Fig. 27a). Most of the light scattering is due to the gas space rather than the enclosing gas vesicle wall; the amount of light scattered decreases by 98% (230) or more (Fig. 27a) when gas vesicles are collapsed by pressure. Angular measurements show that gas vesicle suspensions scatter light strongly in forward and backward directions. The minimum intensity of the scattered light occurs at an angle of 105° to the incident beam (252a). The scattered light can be measured directly in a nephelometer to give a relative measure of gas vesicle content; such measurements can be made directly in a pressure nephelometer in which the thick-walled sample tube is connected to a supply of gas under pressure (232). Isolated gas vesicles at a concentration of 1 μl of gas space per ml give a turbidity of 3.8 nephelometric turbidity units (NTU); those clustered into gas vesicles inside cells give a lower value, 2.0 NTU (68), owing to different types of interaction with light.

Gas vesicle suspensions show attenuance in a spectrophotometer because gas vesicles scatter light out of the collimated light beam directed into the photodetector (229). Suspensions of *Anabaena* gas vesicles containing 1 μl of gas space per ml give a pressure-sensitive attenuance of 2.72 $cm^{-1}$ at a wavelength of 500 nm (253). The attenuance of the gas spaces (the difference in spectra obtained with intact and collapsed gas vesicles [Fig. 27a]), varies as $\lambda^{-2.8}$ (252a); this is the result of the way the scattering angle and intensity change with wavelength. Scattering by gas vesicles, with dimensions of 50 to 1000 nm, falls in the region between Mie scattering (for particles of diameter similar to λ) and Tyndall scattering (for particles of diameter much smaller than λ, when the scattering intensity $i$ varies as $\lambda^{-4}$).

**Light interactions with gas vacuoles in cells.** Gas vacuoles, the aggregates of gas vesicles in cells, interact rather differently with light. The change in attenuance after gas vesicle collapse is more uniform over the visible spectrum (49, 68, 192, 225), but the difference spectra show dips at the absorption peaks of cell pigments (Fig. 27b). One cause of these dips is the proportion of light that passes through the spaces between the cells (for discussion of this sieve or package effect, see references 52 and 112). In cases when the difference spectrum dips to a negative value (225), however, it seems that gas vacuoles must direct some light away from underlying pigments. It has been observed that gas vacuoles are distributed throughout the cells of *Anabaena flos-aquae* grown in low photon irradiance but are located at the periphery of cells grown at high irradiance (192). Gas vacuoles must change the direction of the light path through the cell, but it does not necessarily follow that they provide light shielding.

Direct evidence of light shielding in cells has been sought by comparing the rates of photochemical reactions in cells with intact and collapsed gas vesicles. In *Anabaena flos-aquae*, gas vesicle collapse brought about no significant change in the rate of photoinactivation by UV radiation (indicating that no shielding occurs at these wavelengths) but gave an increase of 4% in the rate of photosynthesis at limiting irradiances; shallow layers of dilute suspensions were used to minimize self-shading effects (192). These experiments should be repeated with filaments grown at high irradiance, which form gas vesicles at the cell periphery.

Ogawa et al. (158) measured light absorption by concentrated suspensions of *Microcystis* cells in a cuvette placed at the center of a white reflecting spherical chamber and illuminated by a narrow beam through a narrow aperture. A photocell in

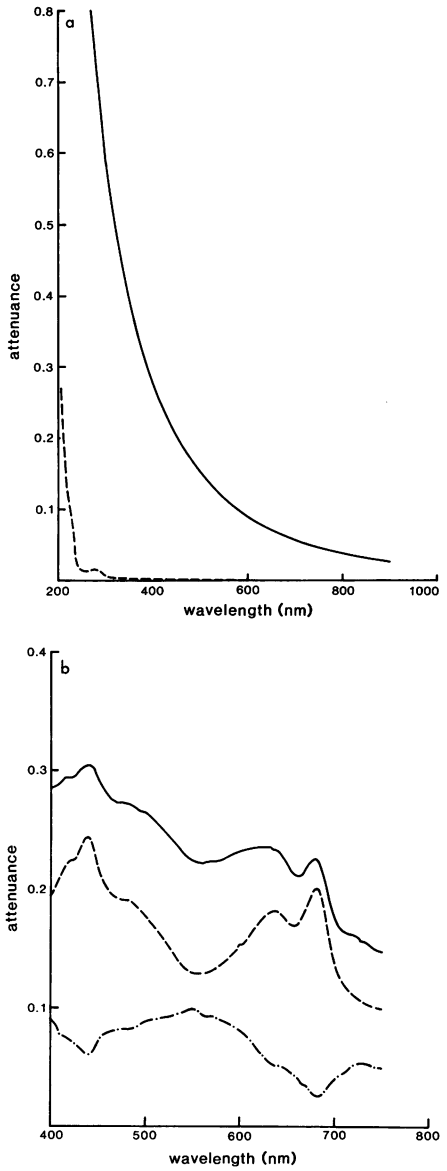


FIG. 27. Attenuation spectra (1-cm path length), due to scattering and absorbance, of suspensions. (a) A suspension, containing 7 μg of gas vesicles $ml^{-1}$ isolated from *Anabaena flos-aquae*, that were intact (——) or collapsed by pressure (– – –). At wavelengths above 350 nm the ratio of attenuance for intact and collapsed gas vesicles is 145:1. The peak at 280 nm is due to absorbance by aromatic amino acids in the gas vesicle proteins. (b) A suspension of *Anabaena flos-aquae* filaments with intact gas vesicles (——) and gas vesicles collapsed by pressure (– – –); the difference spectrum is also shown (– · – · – ·). These spectra are original data, but similar spectra to panel a are shown in references 102 and 229; similar spectra to panel b are shown in references 192 and 225.

this "integrating sphere" received light leaving in all directions from the cuvette, and the total light absorbed by the suspension could therefore be determined. It was expected that if the gas vacuoles shaded pigments in the cells, less light would be absorbed; after gas vacuole collapse there should then be an increase in light absorbed. In fact, slightly more light (about 6%) was absorbed by suspensions of cells with intact gas vesicles than by suspensions with collapsed ones (158). The probable explanation for this is that multiple scattering by the gas vesicles in the concentrated cell suspensions caused an increased path length (68). Since the amount of increased absorption due to multiple scattering is unknown, it is not possible to distinguish separately the possible decrease in absorption due to shielding. These experiments should be repeated with more dilute suspensions in which there is little secondary scattering.

**Measurements of light scattering by gas-vacuolate cell suspensions.** Estimates of the relative gas vesicle content of cell suspensions have been made by measuring the relative turbidity change with a pressure nephelometer (232): measurements are made of the turbidity of the initial suspension ($T$), the suspension after application of pressure causing some gas vesicle collapse ($T_b$), and after collapse of all the gas vesicles ($T_c$). The percentage of gas vesicles collapsed by a given pressure is $\%C = 100\,(T - T_b)/(T - T_c)$. A plot of percent gas vesicle collapse versus pressure—a collapse-pressure curve—is shown in Fig. 20. From this critical pressure distribution can be determined the median critical pressure, at which 50% of the gas vesicles are collapsed, and the mean critical pressure (for the method of calculation, see reference 254). The method has been used widely for investigating changes in gas vesicles and cell turgor pressure (see, e.g., references 110, 119, 123, 129, and 239).

A convenient though arbitrary estimate of the gas vesicle content of cells can be obtained by comparing the turbidity due to gas vesicles ($T - T_c = \Delta T_a$) with the turbidity due to other cell components ($T_c$). The ratio $\Delta T_a/T_c$ has been referred to as the relative gas vesicle content (RGV). It has been used to investigate gas vesicle changes in cultures grown under different conditions (see, e.g., reference 19) and re-formation of gas vesicles after collapse (see, e.g., reference 234). The ratio is affected not only by changes in gas vesicle content, however, but also by changes in the amount of other refractile granules (e.g., cyanophycin or polyphosphate granules) that affect the residual cell turbidity ($T_c$). A better estimate of gas vesicle content is obtained by relating $\Delta T_a$ to some other estimate of biomass, such as protein (142) or ash-free dry mass.

Various modifications of the pressure nephelometer have been described (232, 249). A small portable pressure nephelometer for field work is described by Walsby et al. (249, 260). In commercial nephelometers (Evans Electroselenium Ltd.), light is focused up the tube from below and the light scattered at angles between 50° and 130° to the tube axis is reflected by a curved mirror onto a photocell (232). With this instrument I have observed RGV values exceeding 8.5 in *Prosthecomicrobium* cultures (234), 6 in *Ancylobacter* cultures (252a), and 5 in cyanobacteria (43). Kock and Pinette used a modified spectrofluorimeter in which the scattered light was measured at 90° to the incident beam, an arrangement that should favor the proportion of light scattered by gas vesicles rather than the cells, but the RGV value obtained was only 3.6 in *Ancylobacter* cultures (123).

Dubelaar et al. (49) made measurements with a flow cytometer using an argon laser (wavelength, 488 nm) and showed that the intensity of light scattered perpendicular to the incident beam was 10-fold higher from individual *Microcystis* cells with intact gas vesicles than from those with collapsed ones. The forward-scattered light, on the other hand, increased fivefold when the gas vesicles were collapsed. It was concluded that the gas vesicles decreased the forward scattering because they lowered the average refractive index of the cell and caused a decrease in the phase shift factor, ρ.

**Light scattering by gas-vacuolate cell suspensions and water-blooms.** The presence of particles in a suspension can affect

light absorption in two ways: by backscattering they may reduce the amount of light that penetrates the suspension, and by increasing the length of the light path they increase the attenuation (112). In these ways gas vesicles will cause shading in cell suspensions whether or not they provide shielding within individual cells. Measurements made in well-mixed layers of cyanobacterial suspensions, approximately 10 cm deep, indicate that gas vesicles in the cells decrease the downwelling irradiance by about 13% and decrease the irradiance over all angles by about 29% (see Fig. 2.2 of reference 220). These shading effects would explain why, in concentrated *Microcystis* suspensions illuminated at limiting photon irradiances, the photosynthetic rate was found to increase, by between 7 and 30%, when gas vesicles were collapsed (179).

Gas vacuoles in cells also contribute to light attenuation by cyanobacteria in lake waterblooms. Ganf et al. (68) found that the gas vesicles in colonies of *Microcystis aeruginosa* accounted for 80% of the light scattering in the water column of an Australian reservoir. The increased attenuation by gas vesicles in the near-surface water layers plays a part in the competition between planktonic cyanobacteria and other nonbuoyant phytoplankton (see Role of Buoyancy in Cyanobacteria, below).

## PHYSIOLOGY OF BUOYANCY REGULATION AND GAS VESICLE REGULATION

Gas vesicles provide cells with buoyancy and are also involved in regulating buoyancy (231). Dynamic buoyancy regulation may permit microorganisms to position themselves near a particular depth in a lake or to perform vertical migrations.

### Density of Bacterial Cells

Much of the stuff of living cells has a density greater than that of water (998 kg $m^{-3}$ at 20°C): the density of protein is approximately 1,330 kg $m^{-3}$, that of carbohydrate is 1,550 kg $m^{-3}$, that of nucleic acid is >1,660 kg $m^{-3}$, and that of glycolipid is 1,050 kg $m^{-3}$ (212). A consequence of this is that most cells sink. Some phytoplankton cells may become buoyant by accumulating hydrocarbons, triglycerides, or (in seawater) solutions of "light" ions (95, 197, 265), but none of these buoyancy aids has such a low density as the gas vesicle.

**Measurement of cell density.** The density of cyanobacteria (after gas vesicle collapse) has been measured by centrifuging them in density gradients of the silicone-covered colloid Percoll. The cells band in the gradients at their isopycnic density (159). Filaments of *Oscillatoria agardhii* have a density of 1,076 to 1,093 kg $m^{-3}$ (215). The density of *Anabaena flos-aquae* ranges between 1,025 and 1,036 kg $m^{-3}$ because the trichomes are covered by a sheath, of density 1,000 kg $m^{-3}$, that occupies 58% of the total filament volume (162); without the sheath the trichomes would have had a density range of 1,060 to 1,086 kg $m^{-3}$.

The density of cells that are positively buoyant with intact gas vesicles cannot be measured in the same way because they float above the Percoll density gradient. Their density can be determined by collapsing measured proportions of the gas vesicles and then extrapolating to find the density of the original gas-vacuolate cells (260). Densities of 975 and 992 kg $m^{-3}$ have been found in gas-vacuolate filaments of two *Anabaena* species (260, 261); after allowing for the volume of the sheath, it is calculated that the densities were of the corresponding cells were 942 and 975 kg $m^{-3}$, respectively.

**Amount of gas vesicles required for buoyancy.** The proportion of the cell volume that the gas vesicles must occupy to provide buoyancy varies from about 3 to 10%, depending on the density of the gas vesicles and the other cell constituents (162, 212, 215). Take, for example, the cells of *Anabaena flos-aquae*, without gas vesicles or sheath, with a minimum density of 1,060 kg $m^{-3}$ (162): to become neutrally buoyant (i.e., to reach the same density as water), 6.2% of their cell volume must be occupied by gas. Since the *Anabaena* gas vesicle has a ratio of gas space to wall volume of 10.1 and a density of 119 kg $m^{-3}$ (254), to provide this gas space requires 8.1 kg of gas vesicle protein per $m^3$ of cell volume. This represents 4.9% of the total protein content of these cells (165 kg $m^{-3}$) and 2.9% of the dry mass of the cells (278 kg $m^{-3}$) (162). The cost is higher in species with narrower gas vesicles: in *Oscillatoria argardhii* (254) it would require the equivalent of 7.0% of the cell protein to provide neutral buoyancy.

Cells often have substantially more gas vesicles than are needed for neutral buoyancy (85, 160, 162, 212, 215). In *Anabaena* species, for example, they may have gas space equivalent to 9.8% of the cell volume and gas vesicle protein equivalent to 7.1% of the total protein or 4.2% of the dry mass (162). The buoyancy provided by gas vesicles evidently has a substantial cost in terms of the protein resources of the cell.

### Regulation of Gas Vesicles and Buoyancy in Planktonic Cyanobacteria

Buoyancy regulation of planktonic cyanobacteria in response to light was first demonstrated in cultures of *Anabaena flos-aquae*; buoyancy was gained at low irradiance (228) and lost at high irradiance (46, 230). It was suggested that this response might explain how cyanobacteria positioned themselves on the vertical light gradient in lakes (228) or performed diel vertical migration to and from the lake surface (229) and that it might be important in waterbloom formation (63, 64).

Buoyancy loss at high photon irradiance (photon flux density) has since been demonstrated in a number of planktonic cyanobacteria including some species of *Anabaena* (46, 110, 162, 201, 230, 266), *Aphanizomenon* (128, 129), *Dactylococcopsis* (268), *Gloeotrichia* (262a), *Microcystis* (130, 142, 212, 213, 263), *Oscillatoria* (215, 262, 267), and *Trichodesmium* (223). The mechanisms of buoyancy regulation, summarized here, involve changes in gas vesicles and dense cell components (Fig. 28); they have been reviewed in detail elsewhere (120, 126, 185, 187, 248, 250).

**Quantitative determination of the cause of buoyancy change.** The increase in density of a cell at high photon irradiance could, in theory, be explained by either a loss of gas vesicles or a gain in substances denser than water, such as carbohydrate or protein (231). In a number of the early studies, correlations were found between the loss of buoyancy and either loss of gas vesicles (5, 46, 77, 128) or a rise in carbohydrate content of the cells (142, 222), but in none of these studies was it established that these changes were the cause of the buoyancy change.

The first quantitative analysis of what caused the density change was made by Oliver et al., drawing up a balance sheet of the ballast contributed by each of the major classes of compounds, such as protein, carbohydrate, and lipid (161, 162). Analyses were made of the mass, $M_c$, and density, $\rho_c$, of each of these components; its volume was then given by $V_c = M_c/\rho_w$. The mass of water, of density $\rho_w$, it displaced was calculated as $M_w = V_c\rho_w$, and the mass of the component not supported by water, referred to as the ballast mass, was then $M_c - M_w$. The sum of all the values of $M_c - M_w$ gave the total ballast mass of the cells. There was an independent check on the result obtained: the total ballast mass divided by the cell

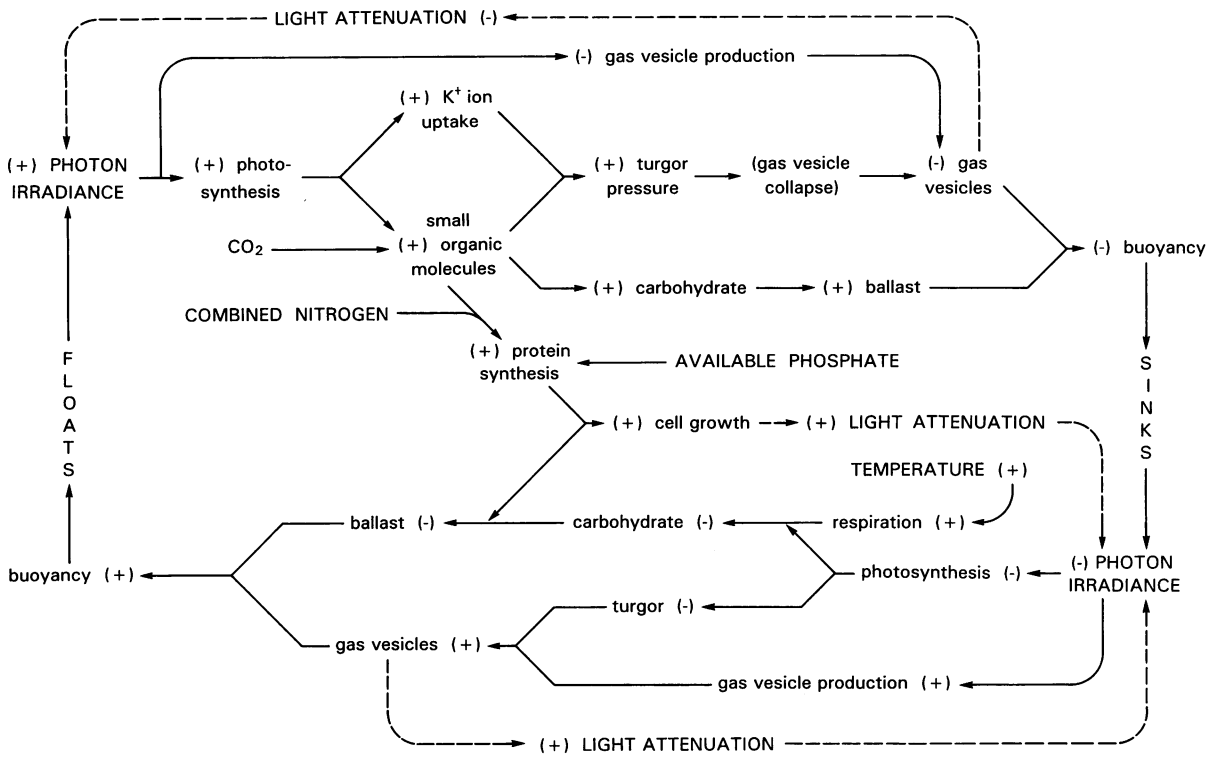


FIG. 28. Summary of the factors involved in the regulation of gas vesicle content and buoyancy in planktonic cyanobacteria. The signs indicate whether the factor increases (+) or decreases (−) as a result of the change (+ or −) in the preceding factor. Redrawn from reference 250 with permission from the publisher.

volume was equal to the excess density of the cells (which was determined by centrifugation on Percoll gradients [162]).

The negative ballast mass of the gas vesicles was calculated in the same way from the volume of gas, $V_g$, determined by using the compression tube (Fig. 19). Since the density of the gas is 1 kg $m^{-3}$, the excess density of the gas is $-997$ kg $m^{-3}$ and the ballast mass of the gas is $(-997V_g)$ kg $m^{-3}$. The cells will float if this negative ballast of the gas exceeds the sum of the (positive) ballast masses of the other components and the cells will sink if it does not.

Ballast balance sheets have been used to analyze which components cause buoyancy loss in cyanobacteria exposed to high photon irradiance. In *Anabaena flos-aquae* the rise in carbohydrate ballast is the normal cause (110), unless the cells are initially very buoyant with gas vesicles, when some gas vesicle collapse may be needed (162). In *Oscillatoria agardhii* there is a cessation of gas vesicle production at high irradiance, so that as the cells continue to accumulate carbohydrate and protein as they grow, their buoyancy is lost (215). In *Microcystis* sp. the cells continue to produce gas vesicles at high irradiance but increased carbohydrate production causes buoyancy loss (212, 213). Carbohydrate accumulation also causes buoyancy loss in *Aphanizomenon flos-aquae* when cells are exposed to increased irradiance or phosphate limitation (129).

Changes in the RGV of cells inferred from measurements of pressure-sensitive turbidity related to cell biomass (43, 46, 118, 119, 125, 130) have been correlated with buoyancy loss, but they cannot be used to determine the quantitative effect of the change on cell buoyancy.

**Regulation of gas vesicle production.** The only direct information on the regulation of *gvp* gene expression of a planktonic cyanobacterium is in a *Pseudanabaena* species whose gas vesicles formed only in low photon irradiance. Damerval et al. (35) have shown by Northern hybridization that *gvpA* expression is light dependent in this organism. When it was grown at a low photon irradiance (5 μmol $m^{-2}$ $s^{-1}$) a single *gvpA* transcript of about 0.4 kb was produced. After transfer to higher irradiance (50 μmol $m^{-2}$ $s^{-1}$), the amount of this transcript decreased, and after 12 h it was undetectable. When the culture was returned to low irradiance, the *gvpA* transcript reappeared after 6 h and reached its original value after about 12 h (35). *Pseudanabaena* species are characterized as cyanobacteria in which gas vesicle production is restricted to the ends of the cells (as in *Oscillatoria redekei* [272]), and these filaments rarely become positively buoyant in culture.

Regrettably, there is no similar information on *gvp* gene expression in any of the important waterbloom-forming cyanobacteria; in some cases this is due to the difficulties in cloning the multiple copies of *gvpA* that exist in these organisms (82a). There have been several studies documenting the outcome of gas vesicle regulation, but they have provided little evidence of the mechanism of the process.

In *Anabaena flos-aquae*, which achieves neutral buoyancy at a low photon irradiance (<13 to 22 μmol $m^{-2}$ $s^{-1}$), gas vesicle content increases at low irradiance (255), but it is uncertain whether this is caused by an increase in the rate of gas vesicle formation because some gas vesicles may be collapsed by rising turgor pressure at high irradiance in this organism (110, 162). In *Aphanizomenon flos-aquae*, gas vesicle content similarly increases at low photon irradiance; Konopka et al. (129) used light-limited chemostat cultures to demonstrate that the production rate of new gas vesicles did not keep pace with the increased growth rate when the photon irradiance was increased from 10 to 30 μmol $m^{-2}$ $s^{-1}$. Buoyancy loss occurred

through the combined effects of this dilution of gas vesicle content by growth and the increase in carbohydrate content of cells.

In *Oscillatoria agardhii* there appears to be no further increase in gas vesicle content after transfer of the cyanobacterium to high photon irradiances (215); dilution by growth might therefore occur more rapidly. Neutral buoyancy is achieved at a lower photon irradiance (only 5 $\mu$mol $m^{-2}$ $s^{-1}$) in this organism, which is adapted to growth in the dim light of the metalimnion of deep lakes (216).

*Microcystis* cells continue to form gas vesicles at high photon irradiance (150 $\mu$mol $m^{-2}$ $s^{-1}$), but they lose buoyancy through increased accumulation of carbohydrate (212). The optimal photon irradiance for production of new gas vesicles is 35 $\mu$mol $m^{-2}$ $s^{-1}$; some gas vesicle production occurs in darkness but only if the cells have been pretreated at high irradiance to allow the accumulation of energy reserves (43). These results are relevant to the recovery of buoyancy in cells or colonies that have sunk into the darkened depths of a lake. In another strain, *Microcystis aeruginosa*, maximum gas vesicle formation occurs at intermediate light-limited growth rates in chemostat cultures (138).

For three of these organisms (*Anabaena*, *Aphanizomenon*, and *Microcystis* spp.) it has been shown that gas vesicle content increases when the phosphate concentration is increased (19, 130, 139). As with the response to light, the mechanism for this change is not known. Studies on cultures of *Oscillatoria rubescens* indicate that the increase is also dependent on the availability of combined nitrogen (119). It is possible to make sense of the ecological benefits that these responses will bring to planktonic cyanobacteria, by regulating their buoyancy in lakes (see Buoyancy Regulation by Cyanobacteria in Lakes, below), but at present there is little information on what actually controls gas vesicle production in cells. At the root of gas vesicle formation must be the expression of the gas vesicle genes. Changes in the ratio of gas vesicle volume to cell protein mass (129, 137, 139) suggest that the rate of expression of the *gvp* genes is modulated.

**Gas vesicle collapse by turgor pressure.** It was first demonstrated in *Anabaena flos-aquae* that a rise in turgor pressure can cause collapse of the weaker gas vesicles in the cells (46, 230). After exposure to high photon irradiance, the turgor pressure exceeded the critical pressures of the weaker gas vesicles originally present; after the turgor rise, only the stronger gas vesicles remained. A reduction in gas vesicle content was indicated by a decrease in the pressure-sensitive turbidity of the cells (46). The turgor pressure, which may rise by as much as 0.24 MPa (2.4 bars), can cause collapse of over 50% of the gas vesicles in this organism, although it may take 16 h of continuous illumination to achieve this degree of loss (110). There is evidence that gas vesicle loss under turgor pressure can also occur in *Aphanizomenon flos-aquae* (128, 129), *Gloeotrichia echinulata* (4a), and *Nostoc muscorum* (7).

Gas vesicle collapse by turgor pressure provided the first explanation of how a cyanobacterium might lose buoyancy at high irradiance; before other mechanisms were discovered, it was assumed to explain the light-stimulated buoyancy loss encountered in other species (118, 184, 186) and particularly when it was demonstrated that there was an accompanying turgor rise (184, 262). Since turgor pressures rarely rise above 0.55 MPa (110, 239), however, this method of gas vesicle regulation can operate only in cyanobacteria that have relatively weak gas vesicles, e.g., certain *Anabaena*, *Aphanizomenon*, *Gloeotrichia*, and *Nostoc* species. Even in these organisms, it has yet to be convincingly demonstrated that collapse of gas vesicles by turgor rise is important in buoyancy regulation of natural populations.

In organisms with weak gas vesicles there are circumstances in which the cells may become so overbuoyant with gas vesicles that the "turgor collapse" mechanism would provide the only way in which buoyancy loss could occur (261). In the majority of cases, however, the accumulation of carbohydrate ballast alone can account for buoyancy loss.

**Accumulation of carbohydrate ballast.** Probably all cyanobacteria accumulate stores of carbohydrate by photosynthesis at high photon irradiance and deplete these stores by respiration and conversion to protein at low irradiance (and at night). Because the carbohydrate is considerably denser than water (and protein), such changes will inevitably cause parallel changes in cell density; in a number of gas-vacuolate cyanobacteria they explain the loss of buoyancy at high irradiance and recovery of buoyancy at low irradiance. In these organisms, background buoyancy provided by gas vesicles is moderated by changes in carbohydrate content.

The role of carbohydrate changes in buoyancy regulation has been demonstrated by quantitative analysis of the cell ballast in species of *Oscillatoria* (161, 215), *Microcystis* (130, 212), *Aphanizomenon* (129), and *Anabaena* (110, 266). Correlations between buoyancy loss and carbohydrate increase have also been demonstrated in other studies (142, 221, 222), and this mechanism is probably widespread in planktonic cyanobacteria.

*Microcystis* cells cultured on a daily light-dark cycle showed a diel pattern of buoyancy change: the cells lost buoyancy during the 8 h of light owing to carbohydrate formation but recovered buoyancy by the end of the dark period, when the carbohydrate was metabolized. This recovery of buoyancy in the dark occurred when the cells were kept at 20°C but not at 8°C, presumably because of the slower metabolism of carbohydrate at the lower temperature (213). The failure to recover buoyancy at low temperatures provides one explanation for the autumnal disappearance of waterbloom cyanobacteria from lakes (160, 213).

## Gas Vesicle Formation in Hormogonia of Cyanobacteria

Certain filamentous cyanobacteria form hormogonia, which act as dispersal stages in the life cycle of the organisms. Hormogonia are short, narrow filaments formed either by the germination of an akinete (nonmotile spore) or by the differentiation and fragmentation of the vegetative filament. In some species the hormogonia are motile and disperse by gliding away from the parent colony, whereas in others they form gas vacuoles and may disperse by floating away.

The first descriptions of gas vacuole formation in hormogonia were those of Canabaeus (26), whose drawings of *Tolypothrix rivularis*, *Calothrix epiphytica*, and *Anabaena variabilis* (now *Nostoc* sp.) clearly depict gas vacuoles forming in differentiating cells. She drew attention to the fact that such gas vacuole formation by *Nostoc linkia*, which occurred in peat bogs, had no connection with waterbloom formation. Her observations that changes in salt concentration caused gas vesicle formation prompted later investigations of gas-vacuolate hormogonia of *Nostoc muscorum* (7, 224) and *Calothrix* sp. (36, 210).

In *Nostoc muscorum* the production of gas-vacuolate hormogonia was induced in cultures diluted with water (224) or when the major salt, $NaNO_3$, was removed from the culture medium (7). The production was also induced by increasing the light intensity (225), which acted synergistically with changing the salt concentration (7). The gas vesicle content of the cells reached a peak 60 h after induction and then declined; the

gas vesicles disappeared over the next 24 h. Collapse of gas vesicles under turgor pressure (see Gas Vesicle Collapse in Cells: Turgor Pressure, above) may be involved in this disappearance (7).

In *Calothrix* sp. the production of gas-vacuolate hormogonia was induced by simultaneously diluting the culture with water and transferring it from white light to red light (37). In the first 2 to 9 h, cells divided without elongation; trichome fragmentation led to hormogonium release at 24 h. Gas vesicle formation occurred between 3 and 24 h. After 3 to 4 days at room temperature, the cells lost their gas vesicles and regained their original vegetative morphology. The number of hormogonia formed decreased when the red light was supplemented with green light, and no differentiation of hormogonia occurred in green light alone (36).

Hybridization with strand- and gene-specific DNA probes showed that during hormogonium production mRNA transcripts were produced from (i) *gvpA1*; (ii) *gvpA1* and *gvpA2*; (iii) *gvpA1*, *gvpA2*, and *gvpC*; and (iv) antisense mRNA covering all of *gvpA2* and part of *gvpA1* (Fig. 4). The antisense mRNA would have been able to form a homologous duplex with the other transcripts and prevent their translation (33). The first transcripts of the *gvp* genes were found at 2 to 3 h after induction by red light. Transcripts of the genes and the antisense RNA reached a maximum after 6 h; the amount of the large transcripts decreased by 12 h, and the smaller ones disappeared by 24 h. Transcription of the *gvp* genes was stopped prematurely by exposure to green light 3 h after induction by red light (36).

There has been no quantitative study of the buoyancy changes of hormogonia. The dispersal of buoyant hormogonia might be important in cyanobacteria that dwell in soils, muds, or rice paddies. The response to dilution might indicate that in nature hormogonia would form after flooding or rainfall. Hormogonia buoyant with gas vesicles might float away in the floodwater. Enough speculation; observations are needed.

### Gas Vesicle Regulation in Other Bacteria

**Regulation in heterotrophic bacteria.** Van Ert and Staley (219) isolated a gas-vacuolate strain (M1) of the ring-forming heterotrophic bacterium *Ancylobacter* (*Microcyclus*) *aquaticus*, in which the number of gas vesicles produced changed markedly in different media. Konopka (131) showed that gas vesicle production was completely inhibited by L-lysine at concentrations as low as 3 μM. L-Threonine and L-cysteine caused partial inhibition; these amino acids are produced on a branch of the same biosynthetic pathway as L-lysine, and their addition may cause its accumulation in cells (40).

Mutant strains that produced gas vesicles constitutively (i.e., even in the presence of lysine) were isolated (11, 132). The genes necessary for gas vesicle production could be transferred on broad-host-range plasmids from these strains into other mutants that had lost the ability for gas vesicle production ($Ves^-$). In 90% of those which regained the $Ves^+$ phenotype, lysine inhibited gas vesicle production. This indicated that gene loci for gas vesicle production and gas vesicle regulation by lysine were not strongly linked (11).

**Regulation in phototropic bacteria.** Pfennig and Cohen-Bazire (175) commented that gas vacuoles were formed by the green sulfur bacterium *Pelodictyon clathratiforme* when grown in dim light and low temperature (4 to 8°C) but that none were detected in cells grown at room temperature. In a brown strain (*P. phaeoclathratiforme*), gas vacuole formation was not affected by temperature (165) but was sensitive to light; gas vesicles were formed only at photon irradiances lower than 5 μmol $m^{-2}$ $s^{-1}$ (164). The purple sulfur bacterium *Amoebobacter purpureus*, isolated from the saline hypolimnion of a meromictic lake, accumulated gas vesicles in darkness and lost them at high photon irradiance (200 μmol $m^{-2}$ $s^{-1}$). The cause of the gas vesicle loss is unknown, but it could not be accounted for by turgor pressure rise. The cells lost buoyancy at high irradiance partly because of the decrease in gas vesicle content and partly because of an increase in carbohydrate content.

It appears that these photosynthetic bacteria may use a parallel mechanism of buoyancy regulation to that in cyanobacteria, accumulating carbohydrate at higher irradiances to offset the buoyancy provided by gas vesicles, although the threshhold irradiance for buoyancy loss is lower (164). Such responses might enable these organisms to regulate their vertical position in the dimly illuminated hypolimnia of stratified lakes (163).

## ECOLOGICAL STUDIES ON GAS-VACUOLATE ORGANISMS

There is an ecological aspect to the function of every biological structure. It is in the natural environment that the structures have arisen and been molded by natural selection, and it is in natural ecosystems that the functions they provide have determined the success of organisms.

The buoyancy provided by gas vesicles will cause a microorganism to sink less quickly or to float upward. Some planktonic microorganisms, e.g., the prochlorophyte *Prochlorothrix hollandica* and the cyanobacteria variously classified as *Oscillatoria redekei* (154), *Pseudanabaena* sp. (189), and *Limnothrix* sp. (152), appear to have too few gas vesicles for positive buoyancy, and it may be that the gas vesicles simply reduce their sinking rates and so increase the probability of their remaining suspended in the water column. A microorganism with sufficient gas vesicles for buoyancy may float out of a benthic layer of mud into the overlying water (29, 30, 233), it may float to the water surface (114), or it may float up to a layer below the surface (228). The buoyancy provided can therefore provide a means of vertical positioning in natural water bodies, if this is permitted by the stability of the water column. The chemical and physical conditions change with depth in a lake, and organisms may benefit if they are able to move up or down such vertical gradients.

Gas-vacuolate organisms may change their buoyancy during the day and perform vertical migrations. The velocity and extent of vertical movements of microorganisms by sinking or floating are strongly size dependent; according to the Stokes equation, for a sphere the velocity varies as the square of the radius. A consequence of this is that single cells or small filaments will move up or down only a few centimeters per day (267), whereas large colonies may move tens or even hundreds of meters (237, 265).

### Role of Buoyancy in Cyanobacteria

The importance of gas vesicles in providing buoyancy for planktonic cyanobacteria in freshwater lakes, oceans, and brine pools has been investigated in some detail. Only a summary is given here because other reviews on this subject are available (120, 185, 187, 188, 265).

In natural waters the light (photon irradiance) decreases exponentially with depth owing to absorption and scattering by the water and substances dissolved or suspended in it (112). Net growth of cyanobacteria and other photosynthetic organisms occurs only if they spend sufficient time above the critical

depth where photosynthesis only just compensates for respiratory losses.

Buoyant cyanobacteria that float up in the intermittently mixed epilimnion of a lake spend more time near the water surface and receive a higher light dose than do other small-celled phytoplankton that circulate within the epilimnion (93, 97, 188, 190, 281), and in this way they outcompete them. The gas-vacuolate cyanobacteria also tend to shade out the phytoplankton lower in the water column, a competitive strategy used by "canopy species" in terrestrial habitats. As discussed above (see Light Scattering by Gas-Vacuolate Cell Suspensions and Waterblooms), light scattering by the gas vesicles in the cyanobacteria can contribute significantly to this shading effect (68). The floating cyanobacteria might also derive other advantages from their proximity to the water surface (such as the increased availability of $CO_2$ diffusing in from the overlying atmosphere [19, 168, 229] and the availability of nitrogenous compounds there [149]), but competition for light is probably of greatest importance.

There are several genera of cyanobacteria that use their buoyancy to occupy the euphotic zone of the epilimnion. In lakes with a shallow euphotic zone and stable epilimnion, one finds *Oscillatoria* species (or the heterocystous *Anabaena* species when nitrogen is limiting [266]) that occur as separate filaments, which circulate within the surface mixed layer; because of their small size the filaments float up slowly and rarely form thick scums. In wind-exposed lakes, colony-forming *Anabaena*, *Aphanizomenon*, *Gloeotrichia*, *Coelosphaerium*, and *Microcystis* species are found. These faster-floating colonial forms rise up more quickly toward the surface during intermittent periods of calm (97, 188). Over long periods of calm, however, there may be disadvantages in floating near the water surface (63, 64). Prolonged exposure to the high irradiance at the surface may cause photooxidation (1), and the concentrated accumulations of cells may lead to rapid depletion of nutrients, such as phosphate (191). By losing buoyancy in response to high irradiance, however, the colonies may sink down and avoid photooxidative damage (185, 188).

**Buoyancy regulation by cyanobacteria in lakes.** Some planktonic cyanobacteria form layers in stratified lakes whereas others execute diel migration. These two phenomena can be explained by the same sort of buoyancy regulation behavior by organisms of different sizes. Stratification is performed by slowly moving separate filaments in clear, oligotrophic lakes. In response to the vertical light gradient during the daytime, filaments nearer the water surface lose buoyancy whereas those deeper on the gradient gain buoyancy, and they converge on a depth ($z_n$) in the metalimnion where the average irradiance supports neutral buoyancy (267). The filaments become more buoyant at night, but because of their small size they move up only a few centimeters before the next day (262, 267, 268). In this way certain *Oscillatoria* spp. (10, 117, 125, 127, 267, 280) and *Limnothrix* spp. (153) may form metalimnetic populations that persist for several months each summer in deep, well-stratified lakes. This sort of stratification can occur only in relatively transparent lakes, where the light penetrates to a sufficient depth that $z_n$ is below the surface water layers that are mixed by wind or by nighttime convectional cooling. In less transparent eutrophic waters, where $z_n$ is much nearer the surface, the population is regularly dispersed by mixing and cannot stratify (266).

It has been demonstrated that buoyancy regulation is the mechanism by which filaments stratify in metalimnetic layers; filaments suspended in containers above the layer sink down, while those suspended below it float up (262, 267). Evidence of the direction of movement in the lake water can be collected by using upward- and downward-facing traps (125).

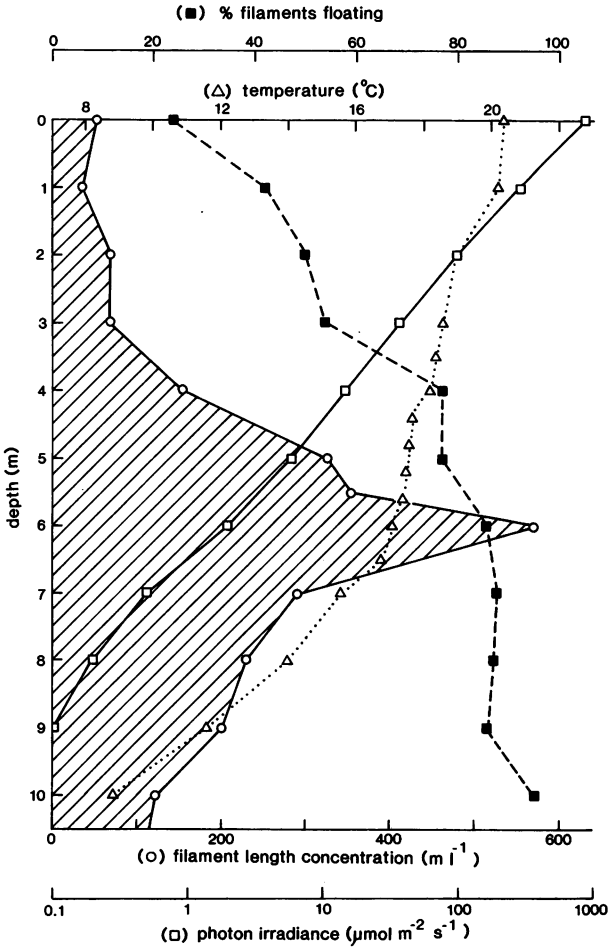


FIG. 29. Vertical distribution and buoyancy state of a population of *Oscillatoria aghardii* filaments in lake Gjersjøen, Norway, in relation to temperature and photon irradiance. Redrawn from Walsby et al. (267) with permission from the publishers.

By the same buoyancy-regulating mechanisms, faster-moving colonies may migrate down below the metalimnion during the day and then return to the water surface at night (128, 263). This behavior can account for waterblooms that appear at a lake surface in the morning and disappear by the afternoon. The same buoyancy-regulating behavior can account for both stratification and vertical migration; this was confirmed by our observations at Lake Gjersjøen in Norway, when some of the *Oscillatoria* filaments from the metalimnion formed colonies that then performed diel migration (Fig. 29) (267). It has been shown by computer modeling techniques that differences in the amplitude of movement can be explained solely in terms of the differences in sinking or floating velocity due to colony size (140, 251).

Colonial cyanobacteria often form surface scums, which may persist for days or even weeks (281). This suggests that buoyancy regulation sometimes fails. Several reasons have been suggested for the failure: the rate at which $CO_2$ is taken up by the concentrated cyanobacteria may exceed the rate at which it is replenished from the air, so that carbohydrate cannot be accumulated (19); photooxidation may damage the cells before they have had time to lose buoyancy (188); buoyant

cells in the middle of the floating colonies may not respond, because they are shaded or prevented from having access to $CO_2$ by the peripheral cells in the colony (167); or the cells may become so highly gas vacuolate that they are unable to counteract the buoyancy by carbohydrate accumulation (261). Ibelings and Mur (96) studied scums formed in the laboratory and showed that owing to the steep attenuation of light, photosynthetic activity was restricted to the upper few millimeters. Although colonies there lost buoyancy, they were kept at the water surface by the blanket of buoyant, photosynthetically inactive colonies below them. The same occurs in thick hyperscums that form in lakes when floating colonies are concentrated by wind action near lee shores (281).

The presence of a persistent surface waterbloom does not necessarily indicate that individual colonies are remaining at the surface for prolonged periods; computer modeling indicates that waterblooms may result from an overlapping succession of colonies that each spend only a short time at the water surface (140).

During prolonged periods of calm weather, colonial cyanobacteria may become becalmed at the water surface and then be further concentrated by drifting to leeward shores (185, 188). These noxious scums arouse interest because of the stench they make, the way they discolor the water, the harm they do to fish, and the toxins they release into water supplies (27). A final consequence of the buoyancy provided by gas vesicles is that when the population finally dies, the morbid remains are left at the water surface rather than sinking out of sight at the bottom of a lake.

Some *Microcystis* populations do sink to the lake bottom at the end of the autumn. In Blelham Tarn in the English Lake District, the floating colonies were weighed down by iron compounds that precipitated when ferrous sulfide dissolved in the anerobic hypolimnion became oxidized as the lake mixed at overturn (160). In Abbots Pool, Avon, England, the *Microcystis* colonies failed to regain their buoyancy at night because the metabolism of dense carbohydrate ceased when the temperature fell below 11°C (213). It is possible that the colonies regain buoyancy in the spring and float up to form the inoculum for the subsequent season (186).

### Role of Buoyancy in Other Gas-Vacuolate Microorganisms

The observation that gas-vacuolate organisms occur almost exclusively in aquatic habitats has been used to support the idea that buoyancy is their principal function (31, 231). Some gas-vacuolate bacteria have been described from aerobic water layers (202, 219) and seawater (100, 204, 205). They are found most abundantly, however, in the anaerobic hypolimnia of stratified lakes (24, 25, 29, 30, 50, 51, 74–76, 89, 124, 146, 195, 196, 233). Surveys of such layers have revealed 20 or more morphologically distinct gas-vacuolate forms in a single lake (29, 233). The gas-vacuolate organisms present include iron bacteria, purple and green sulfur bacteria (24, 25, 76, 233), *Chloronema giganteum* (76) (possibly a green nonsulfur bacterium), and a wide variety of appendaged, filamentous, colonial, sheathed, and other morphological forms, including several consortia in which one or both members contained gas vesicles (Fig. 30; see also Table 3) (see reference 242 for a review).

The changes in vertical distribution of several gas-vacuolate bacteria in Crose Mere, England, were monitored by serial sampling (Fig. 31). They remained confined to the anaerobic hypolimnion for several months, and it was estimated that the buoyancy provided by gas vesicles would have been necessary to sustain these organisms in suspension over this time (30). Each organism formed a population maximum at a different depth and different time. There are two possible explanations for this. First, the gas vesicles may simply have given the organisms approximately neutral buoyancy so that they sedimented or floated only very slowly and had time to develop a population maximum where conditions most favored their growth. Second, the organisms may have regulated their buoyancy in response to some factor that formed a vertical gradient, in the way that cyanobacteria stratify on light gradients. The manner in which these various organisms regulate their positions at different depths would make a fascinating study, but it first requires their isolation and culture. This has been achieved in few instances, with the notable exceptions of certain green and purple sulfur bacteria isolated by Pfennig and coworkers (see reference 176 for a review) and some heterotrophic bacteria isolated by Staley and coworkers (see, e.g., references 99, 204, 205, 218, and 219).

**Photosynthetic bacteria.** The photosynthetic bacteria typically occupy the anaerobic hypolimnia of stratified lakes. Gas vesicles are found in both the purple (members of the *Chromatiaceae* and *Ectothiorhodospiraceae*) and green (members of the *Chlorobiaceae*) sulfur bacteria and the green (but not the purple) nonsulfur bacteria. These groups of organisms harvest the longer-wavelength far-red light that remains unabsorbed by the phytoplankton in the epilimnion. They are often found in discrete layers (circumscribed above by the presence of oxygen or absence of sulfide and below by the absence of light) (29, 54, 163).

Some gas-vacuolate photosynthetic bacteria have been shown (in cultures) to lose buoyancy when the photon irradiance is increased (see Regulation in Phototrophic Bacteria, above); they may use buoyancy regulation to stratify on vertical light gradients in lakes, although there has been no direct demonstration of this. Many of these organisms are killed by exposure to oxygen in the presence of light, and it is therefore important that they do not stray up into the aerated waters of the epilimnion. A mechanism that might prevent this has been observed in *Amoebobacter purpureus*: the dispersed cells aggregate and sink when sulfide is depleted by oxidation (166). Owing to their small size, individual bacteria float up or sediment down only very slowly: Pedrós-Alió and Sala showed that, in Lake Cisó, Spain, no migration could be detected by nonmotile gas-vacuolate cells of *Amoebobacter* sp. although motile flagellate cells of *Chromatium minus* exhibited a daily vertical migration over 35 cm (170).

Eichler and Pfennig (54) showed that a number of gas-vacuolate purple bacteria overwintered in flocs of organic material on the benthic sediments in Schleinsee, a drumlin lake in southern Germany. After the lake stratified in the spring, these bacteria disappeared from the sediment and appeared throughout the hypolimnion. Later they became concentrated toward the top of the hypolimnion. These changes in vertical distribution may depend on the buoyancy provided by gas vesicles. At the autumn overturn, the cells precipitated with oxidized iron and manganese and sank to the sediment, as had been observed with cyanobacteria (160).

**Halobacteria.** There has been little ecological investigation of gas-vacuolate halobacteria. Gas vesicles occur in the genera *Halobacterium* (145, 171) and *Haloferax* (57) and in halophilic square bacteria (240), which may be related to the genus *Haloarcula* (206). These organisms occur in shallow brine pools, among salt-encrusted sabkhas (65), in solar salt evaporation ponds, and in large brine lakes of arid regions. They are aerobic heterotrophs, but under microaerobic and anaerobic conditions they respire anaerobically and produce purple membranes that support a light-dependent proton pump driving photophosphorylation (21).

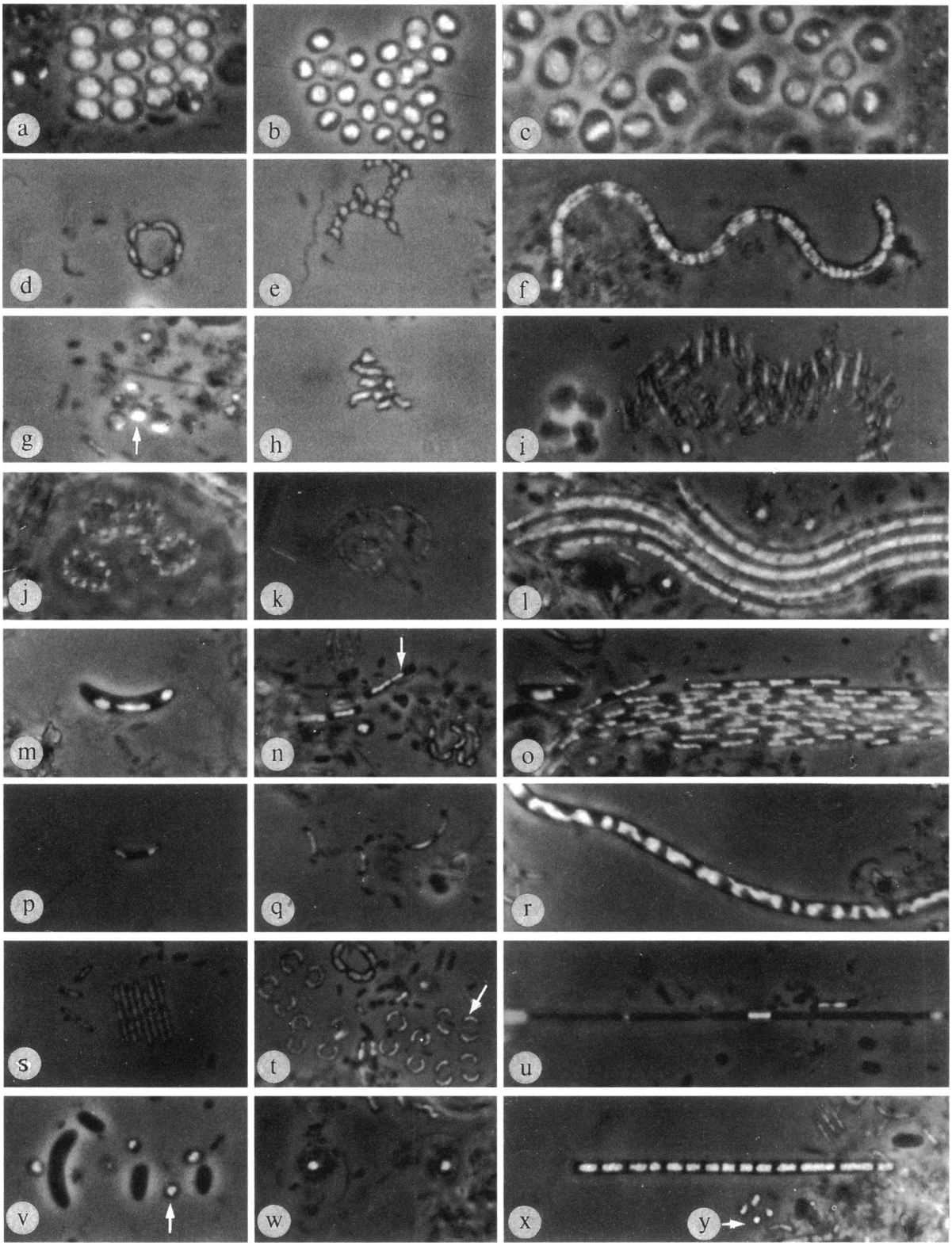


FIG. 30. Phase-contrast micrographs of gas-vacuolate bacteria from stratified lakes, showing a range of morphological forms. Reproduced from Clark and Walsby (29) with permission from the publisher. Magnification, ×1,680.

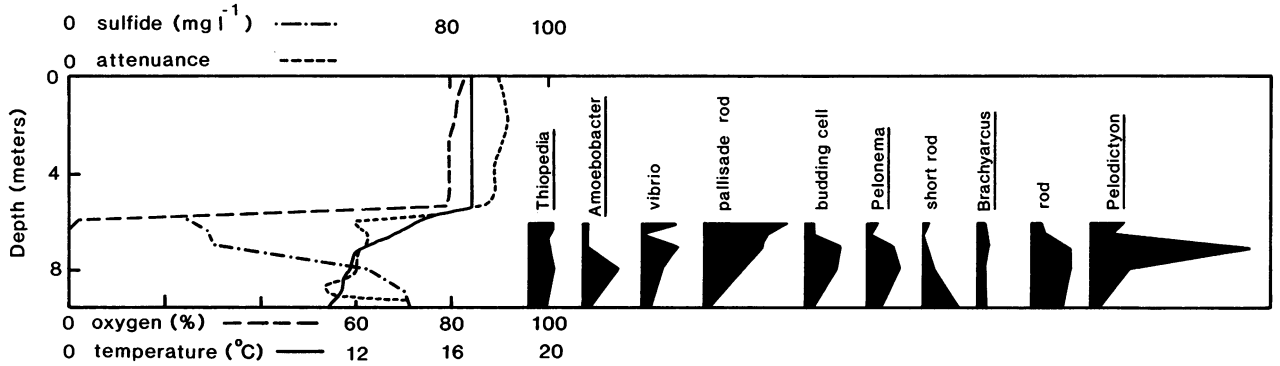


FIG. 31. Vertical distributions of nine gas-vacuolate bacteria in the hypolimnion of a stratified lake (Crose Mere, England), in relation to vertical gradients of temperature, oxygen (percent saturation), sulfide concentration, and relative light attenuation. Modified from Clark and Walsby (30) with permission from the publisher.

In 1932, Petter (171a) suggested that the function of gas vesicles in halobacteria might be to buoy the cells to the surfaces of brine pools, where they would benefit from higher concentrations of oxygen. Oxygen is less soluble in brine than in water, and steep diffusion gradients may therefore develop (145). Light may also be steeply attenuated by precipitated salts. The role of gas vacuoles in the vertical distribution of halobacteria in brine pools that have steep gradients of light, oxygen, and other solutes would make an interesting study, which could draw on the wealth of information on the genetics of these organisms. First, more physiological studies on gas vesicle formation are required; we know only that gas vesicle production increases in the stationary phase of a culture (145) and in high salt concentration (57). In neither case is quantitative information available.

**Heterotrophic bacteria.** Staley and coworkers isolated a number of heterotrophic bacteria, including *Aquabacter spiritensis* (99), the ring-forming bacterium *Ancylobacter* (*Microcyclus*) *aquaticus* (219), and the prosthecate bacterium *Prosthecomicrobium pneumaticum* (202), from the aerobic epilimnia of lakes. In these microorganisms, which are obligate aerobes, it may be that the function of the gas vesicles is simply to buoy the cells to the oxygenated water surface, as proposed for halobacteria, but again there has been no investigation in natural habitats. In static *Prosthecomicrobium* cultures I have found that the gas-vacuolate wild type, which floated to the aerated water surface, outcompeted a gas-vesicle-deficient mutant, which always sank. In shaken cultures (and in special vessels closed top and bottom by gas-permeable membranes in contact with air), the mutant and wild type grew at about the same rate (234). The proportion of the gas-vacuolate wild-type and nonvacuolate mutant was determined by plating on agar and counting the colonies of the two types, which were readily distinguishable (Fig. 32).

It has been mentioned above (see Regulation in Heterotrophic Bacteria) that lysine inhibited gas vesicle production by *Ancylobacter* spp. (131). Konopka (131) suggested that lack of lysine might be an indicator of unfavorable growth conditions and that by producing gas vesicles in response to lysine depletion, cells might be able to migrate to a region of the water column more favorable for growth. Unfortunately, there have been no studies relating buoyancy regulation to the ecology of the organisms in the water bodies from which they were obtained.

Staley and coworkers have isolated two facultative anaerobes that possess gas vesicles, *Ancalomicrobium adetum* (202) and *Enhydrobacter aerosaccus* (204). These organisms tolerate oxygen but grow microaerophilically. The latter was isolated from the oxygen-depleted zone of a eutrophic lake. It would be interesting to know whether these organisms are able to regulate their positions on vertical gradients of oxygen or nutrients.

**Obligate anaerobes.** The role of gas vesicles in extreme anaerobes is curious. Gas vesicles have been found in the methanogenic archaeon *Methanosarcina* sp., which grows only under strictly anaerobic conditions (6, 279). Certain *Clostridium* species produce glistening caps, formed from gas vesicles, adjacent to their spores (135, 136). A similar structure has been found on the spores of *Desulfotomaculum acetoxidans* (274), a sulfate-reducing bacterium that oxidizes acetate under strictly anaerobic conditions (273). These organisms have been isolated from manure, from the rumen, and from muds of dung-contaminated habitats. Do the gas vacuoles buoy up the cells and spores up from muds into the anaerobic hypolimnion or disperse them through soil water?

### Adaptation of Gas Vesicles to Different Pressures

Gas vesicles will provide buoyancy only if they remain intact; gas vesicles in different organisms should therefore be adapted to withstand the pressures they are likely to encounter in their natural habitats. They should not be stronger than necessary,

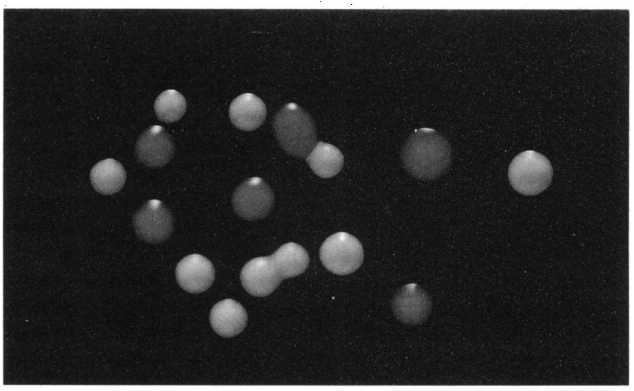

FIG. 32. Colonies of *Prosthecomicrobium pneumaticum* growing on agar: white opalescent colonies of the gas-vacuolate wild type and translucent colonies of the nonvacuolate mutant. Reproduced from Walsby (234) with permission from the publisher.

because, for the reasons already given, this would require them to be narrower and therefore less efficient in providing buoyancy (230, 248). In this section I review the evidence for the expected correlation between gas vesicle critical pressure and pressure in the habitat, by examining examples of different types of water body. Most of the examples involve cyanobacteria, because there has been little investigation of other groups.

As explained in Fig. 15, the net pressure ($p_n$) on a gas vesicle in a cell is given by the expression

$$p_n = p_h + p_t + p_f - p_g \quad (20)$$

In the context of natural habitats, $p_h$, the hydrostatic pressure due to depth ($z$), rises by approximately 0.01 MPa $m^{-1}$; $p_f$, the atmospheric pressure at sea level, is 0.1 MPa (1 bar); and $p_g$ is the gas pressure in gas vesicles, usually the same as $p_f$ (0.1 MPa) although it might be only 0.08 MPa in a hypolimnion lacking dissolved oxygen or even lower if dissolved nitrogen gas had been depleted by nitrogenase activity (268). (Note that the concentration of dissolved air does not increase with hydrostatic pressure due to depth in a lake, and, hence, neither does $p_g$.) The major determinants of $p_n$ are therefore usually $p_h$ and $p_t$, the cell turgor pressure.

For all gas vesicles to survive, their minimum critical pressure must exceed $p_n$. If cells are to float up from depth, sufficient gas vesicles must survive for buoyancy to be retained; the minimum proportion needed varies from 50% to >90%, but the more that are retained the faster the organism will float (160, 212).

**Correlation between pressure in the natural habitat and $p_c$.** I consider here the "case histories" of gas-vacuolate organisms in four types of habitat, in order of increasing pressure.

**(i) Shallow hypersaline pools.** Gas vesicles experience the lowest pressures in halobacteria that inhabit shallow hypersaline pools such as those found in a sabhra (65). The depth is usually less than 1 m ($p_h$ < 0.01 MPa), the cells may have negligible turgor pressure ($p_t$ = 0 MPa) (230, 240), and the saturated brine may become deoxygenated ($p_f - p_g$ = 0.02 MPa); the net pressure across the gas vesicle wall may therefore be no more than $p_n$ = 0.03 MPa. There has been no direct determination of the $p_c$ of gas vesicles in halobacteria from specific pools, but these organisms have gas vesicles with the greatest width and lowest critical pressure, ranging from only 0.04 to 0.16 MPa, with a mean $p_c$ of 0.09 MPa (230). The narrower cylindrical gas vesicles of gas-vesicle-deficient mutants have a higher critical pressure (mean $p_c$ = 0.21 MPa [266a]). They could provide adaptation in deeper brine lakes, but there has been no ecological investigation of this.

**(ii) A brine lake.** Cells of the cyanobacterium *Dactylococcopsis salina* are distributed throughout the depth of Solar Lake, Sinai, which contains brine of density 1,200 kg $m^{-3}$ to a depth of 5 m ($p_h$ = 0.06 MPa). The hypolimnion of the lake becomes anoxic during winter stratification ($p_f - p_g$ = 0.02 MPa). The cells taken from the lake water have a turgor pressure $p_t$ = 0.08 MPa. The gas vesicles therefore experience pressures up to only $p_n$ = 0.16 MPa (268). Critical pressure distributions of gas vesicles from cultured cells indicate a mean $p_c$ = 0.33 MPa (254), whereas the weakest gas vesicles, $p_c$ = 0.18 MPa, are just strong enough to withstand the pressures in the lake. The gas vesicles of this organism are among the weakest and widest of those found in cyanobacteria (254). The cells of *Aphanothece halophytica* from the same lake (268) have gas vesicles of rather similar width and strength (110a).

**(iii) Freshwater lakes.** Many freshwater lakes contain the cyanobacteria *Anabaena*, *Aphanizomenon*, *Gloeotrichia*, *Microcystis*, and *Oscillatoria* spp., in which the mean critical pressures of gas vesicles vary from 0.55 to 1.0 MPa. The lakes may vary in depth from 5 to 70 m or more ($p_h$ = 0.05 to >0.7 MPa). Cell turgor pressures are usually in the range $p_t$ = 0.3 to 0.4 MPa. The potential range of pressure therefore varies from 0.35 to >1.1 MPa, depending on the range of depth encountered. There is evidence that stronger gas vesicles are correlated with cyanobacteria from deeper lakes (216). Three examples are considered here.

In Lake Rotongaio, New Zealand, *Anabaena minutissima* had gas vesicles with a mean $p_c$ = 0.62 MPa; the cells had a maximum turgor pressure $p_t$ = 0.43 MPa, and most of the population were within the top 3 m ($p_h$ = 0.03 MPa); the combined pressure ($p_n$ = 0.46 MPa) was shown to collapse less than 2% of the gas vesicles. Analysis of the critical pressure curves showed that circulation of filaments to the lake bottom (where, for 80% of the lake area, $z$ = 15 m and $p_h$ = 0.15 MPa) would have caused collapse of less than 30% of the gas vesicles in the turgid cells, leaving 90% of the filaments buoyant (266). The rather weak gas vesicles were just strong enough to withstand the pressures in this lake.

In Lake Gjersjøen, Norway, *Oscillatoria aghardii* had gas vesicles with a mean $p_c$ = 1.0 MPa, apparently excessive for filaments present mainly within the top 6 to 10 m of the water column ($p_h$ = 0.1 MPa; $p_t$ = 0.3 MPa; $p_n$ = 0.4 MPa [Fig. 29]) during the summer months. In the autumn, however, overturn occurs and the population is circulated through much of the 70-m depth (when $p_t + p_h$ = 1 MPa). The filaments that remain buoyant with intact gas vesicles are later able to float up under the ice, where they persist during the winter (267). When the ice melts in spring, overturn occurs again and the filaments with intact gas vesicles float up to form the inoculum of the metalimnetic summer population.

It might be wondered whether there is an ideal $p_c$ for each particular lake (although this is tantamount to wondering whether there should be one ideal cyanobacterium in each lake, the sort of problem which has been addressed by Hutchinson in his "paradox of the plankton" [94]). There is evidence against this. The strain of *Anabaena flos-aquae* from Windermere in the English Lake District (18) has gas vesicles with a mean $p_c$ of 0.60 MPa (254), which would generally be adequate to withstand the sum of $p_h$ and $p_t$ encountered in the epilimnion; gas vesicles in *Anabaena lemmermannii* from the same lake, however, have a mean $p_c$ of 0.93 MPa (261). The two species behave differently. The weak gas vesicles of *A. flos-aquae* facilitate buoyancy loss at high irradiance, which might enable it to escape from surface waterblooms by sinking (46, 110). *A. lemmermannii*, with its stronger gas vesicles, cannot do this, and it can become overbuoyant and be stuck at the water surface (261). Its strategy is to form akinetes that lose gas vesicles and sink to the lake bottom (191), at a depth of >40 m in Windermere. The akinetes later germinate to produce hormogonia. For them to float up into the epilimnion, their gas vesicles must be strong enough to withstand the total pressure there ($p_h + p_t$ > 0.8 MPa).

**(iv) Oceans.** Several *Trichodesmium* species have been identified in the oceans (200), where they form extensive surface waterblooms (9). They contain gas vesicles of greater strength than any other organisms, with a mean $p_c$ of 1.2 to 3.7 MPa. *Trichodesmium* colonies have been found at depths exceeding 200 m (28) ($p_h$ > 2.0 MPa); their gas vesicles must withstand these pressures if the colonies are to float back to the surface layers of the sea. The colonies may migrate over such depths during diel buoyancy regulation (141).

This brief survey supports the view that gas vesicles in different organisms have become adapted to withstand the

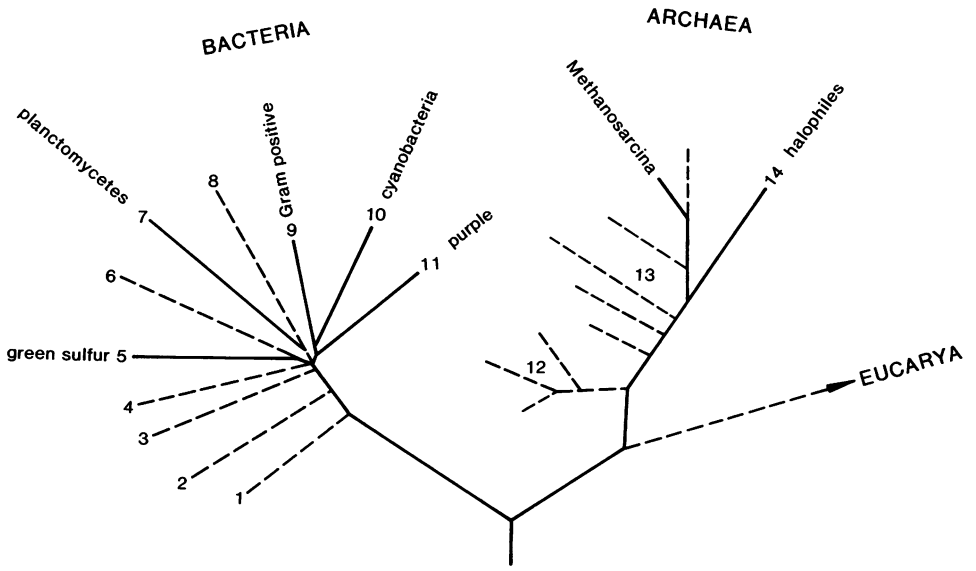


FIG. 33. Part of the universal phylogenetic tree according to Woese et al. (276, 277) showing phyla or groups in which gas vesicles occur (solid lines) and other phyla or groups in which gas vesicles have not yet been demonstrated (dashed lines, but see Table 3). Bacteria: 1, the order *Thermotogales*; 2, green nonsulfur bacteria; 3, deinococci; 4, spirochetes; 5, green sulfur bacteria; 6, bacteroides-flavobacteria; 7, planctomycetes; 8, chlamidiae; 9, gram-positive bacteria; 10, cyanobacteria; 11, proteobacteria (purple bacteria). Archaea: 12, extreme thermophiles; 13, methanogens; 14, extreme halophiles. The lengths of the branches are not directly applicable to particular gas-vacuolate organisms; they refer to other organisms in the same phyla.

pressures they are likely encounter in the natural environment (248). In the following section the mechanism of natural selection which has led to this adaptation is discussed.

## VARIATION AND EVOLUTION OF GAS VESICLES

### "How the Anabaena Got Her Gas Vesicle"

To paraphrase Dobzhansky (47), it is possible to describe gas vesicles without asking questions about their origins but the descriptions acquire meaning and coherence only when viewed in the perspective of evolutionary development. The danger in attempting an evolutionary perspective is that all that will emerge is a "Just So Story": "How the Camel Got His Hump" (111), "How the Anabaena Got Her Gas Vesicle." Nevertheless, there are a number of aspects of the gas vesicle story that are worth telling, and at least some of the speculations are falsifiable by the information emerging on the sequences of gas vesicle genes.

The story of a structure's evolution has three parts, which can be summarized as phylogeny, morphogenesis, and natural selection.

The problem of determining the phylogeny of a structure and of the organisms in which it occurs has been made tractable by protein and nucleic acid sequencing (276). I comment below on the phylogenetic implications of the sequences of gas vesicle proteins and genes from a number of organisms (78, 226). Although phylogenetic studies provide information on the path that has been taken by evolution, however, they provide no direct information on the way in which natural selection has directed that path.

What may appear to be the major unsolved problems of evolution are often the unsolved problems of morphogenesis in ontogeny. The evolution of the hump by the ancestral camel population may have involved minor changes in the genome, but we cannot explain how they were translated into such gross morphological changes, partly because we know so little of the morphogenetic process (81) and partly because we know little of the gene products involved. What was initially so attractive about the gas vesicle was that it appeared to be a very simple structure, formed from a single type of protein (104, 227), i.e., GvpA, which alone might determine the morphology and evolution of the structure. Whether the gas vesicle contains one protein, a minimum of two proteins, or even several, as suggested by the complexity of the *gvp* gene clusters in *Halobacterium* and *Anabaena* spp., it should be possible to describe its molecular structure completely in terms of its protein components. Methods of site-specific mutagenesis could then be used to investigate which parts of the amino acid sequence of the gas vesicle proteins determine differences in shape.

The third part of any evolutionary story concerns the factors responsible for natural selection of particular variants. For positive selection, the changed phenotype must give improved performance either in the same environment or in a different one and so increase the reproductive success of the individual that possesses it. In the case of the gas vesicle, a quantitative account can be given of the two counteracting factors, economy and the ability to withstand different hydrostatic pressures, that have driven natural selection for gas vesicles of different widths. We therefore have elements of the three parts of the story for the evolution of the gas vesicle.

### Phylogeny of Gas Vesicles

**Antiquity or lateral transfer?** Gas vesicles of similar structure occur in at least 5 of the 11 phyla of bacteria, including the green sulfur bacteria, the $\alpha$ and $\gamma$ subdivisions of the purple bacteria (proteobacteria), the cyanobacteria (including the prochlorophytes), the planctomycetes (17, 70), and the gram-positive bacteria (Fig. 33; Table 3). Some of the many other unidentified gas-vacuolate bacteria (242) may be from other phyla. The occurrence of gas vesicles may therefore be as widespread as that of photosynthesis, which Woese (276)

TABLE 3. Genera of gas-vacuolate bacteria and archaea[a]

**BACTERIA**
- Section 3: Nonmotile curved bacteria
  - *Ancylobacter* (= *Microcyclus*) (219), *Meniscus* (98), *Brachyarcus*
- Section 4: Aerobic rods and cocci
  - *Aquabacter* (99) (phylum 11α), *Lampropedia*
- Section 5: Facultatively anaerobic rods; *Vibrionaceae*
  - *Enhydrobacter* (204)
- Section 13: Gram-positive endosporeforming rods and cocci (phylum 9)
  - *Clostridium*, *Desulfotomaculum* (274)
- Section 18: Anoxygenic photosynthetic bacteria
  - Purple bacteria (phylum 11γ-1):
    - *Chromatiaceae*
      - *Lamprobacter*, *Lamprocystis*, *Thiodictyon*, *Amoebobacter*, *Thiopedia*
    - *Ectothiorhodospiraceae*
      - *Ectothiorhodospira* (97a)
  - Green bacteria
    - Green sulfur bacteria (phylum 5)
      - *Pelodictyon*, *Ancalochloris*, *Chloroherpeton* (21), *Clathrochloris*
    - Consortia
      - *Chlorochromatium*, *Chloroplana*, *Cylindrogloea*
    - Filamentous green bacteria (phylum 2 or 5?)
      - *Chloronema*
- Section 19: Oxygenic photosynthetic bacteria
  - Group I: Cyanobacteria
    - Order: *Chroococcales*
      - *Dactylococcopsis*, *Microcystis*, *Coelosphaerium*
    - Order: *Oscillatoriales*
      - *Spirulina*, *Oscillatoria*, *Trichodesmium*, *Pseudanabaena*
    - Order: *Nostocales*
      - *Anabaena*, *Anabaenopsis*, *Aphanizomenon*, *Nodularia*, *Cylindrospermum*, *Nostoc*, *Calothrix*, *Gloeotrichia*
  - Group II: Order *Prochlorales*
    - *Prochlorothrix*
- Section 20: Aerobic, chemolithotrophic, iron-depositing bacteria
  - *Siderocapsa*, *Ochrobium*
- Section 21: Budding and appendaged bacteria
  - *Ancalomicrobium*, *Prosthecomicrobium*, *Stella* (phylum 11α), *Isosphaera* (17, 70) (phylum 7)
- Section 22: Sheathed bacteria
  - *Leptothrix*
- Section 23: Nonphotosynthetic gliding bacteria
  - *Pelonema*, *Peloploca*, *Achroonema*

**ARCHAEA**
- Section 25: Archaea
  - Group I: Methanogens; Order *Methanomicrobiales*
    - *Methanosarcina*, *Methanothrix*[b] (phylum 13)
  - Group III: Extreme halophiles; Order *Halobacteriales*
    - *Halobacterium*, *Haloferax* (58), *Natronobacterium* (155a), square bacterium (240) (phylum 14)

[a] Classified according to *Bergey's Manual of Systematic Bacteriology*. The phylogenetic positions of the groups or genera are indicated by the phylum numbers used in Fig. 34, where known. References are given only to genera not listed in the last edition of Bergey or by Walsby (241, 242).

[b] Gas vesicle content to be confirmed.

concluded must have its origin deep in the bacterial branch of the universal phylogenetic tree. The origins of gas vesicles may be even deeper because these structures also occur in two of the archaeote phyla (203), the extreme halophiles (*Halobacterium* spp.) and methanogens (*Methanosarcina* spp.) (276, 277).

There are homologies in the amino acid sequences of both GvpA and GvpC of cyanobacteria and the archaeote halobacteria. The *gvpA* and *gvpC* genes in these organisms must therefore have had a common origin. They must either have arisen in forms of life ancestral to bacteria and archaea or have been distributed among unrelated organisms at some later date by lateral genetic transfer (203, 226). Clues on which of these paths was taken might be found by comparing the sequences of these proteins in different organisms.

The N-terminal amino acid sequences of GvpA in cyanobacteria, purple bacteria, and halophilic archaea (Fig. 2) show such a high degree of homology that it raises doubts about whether this protein can have been evolving for as long as the time that bacteria and archaea have been separated (276). The number of differences in the sequences does increase, however, with the depth of phylogenetic separation. The lowest homology is between the halobacteria and cyanobacteria (ranging from 54 to 64% identical), and the next lowest is between the purple sulfur bacteria and cyanobacteria (72 to 80%). The range within the cyanobacteria is 79 to 100%, and within this group the highest homologies are within the related groups, namely 98 to 100% within the three heterocystous forms (*Anabaena*, *Aphanizomenon*, and *Calothrix* spp.) and 95 to 98% within the Oscillatoriacean forms (*Oscillatoria*, *Pseudanabaena*, and *Spirulina* spp.), which have a distinctive signature of valine at residue 5 and serine at residue 40 (see the distance matrix in reference 78). The sequences that have diverged farthest from these are those of the two unicellular halophilic cyanobacteria, *Dactylococopsis salina* and *Aphanothece halophytica*, which are identical to one another (78, 110a). Thus the pattern of homology is consistent with the probable phylogeny of these organisms (71) and supports the idea that the high homology results from slow evolution of the gene.

Some evidence about the evolution of GvpC can be gleaned from comparisons of the N-terminal sequences of this protein in cyanobacteria and halobacteria (78), from the repeating motifs in the complete GvpC sequences of three of these organisms (37, 84, 91, 106), and from an analysis of the repeating substructure within these motifs (37) (see below).

The N-terminal sequences of GvpC in seven species of cyanobacteria show much lower homology than those of GvpA in the same organisms (Fig. 7). Within the heterocystous species the range is 51 to 92% identical; *Microcystis* sp. has 42 to 45% of residues identical to this group. In GvpC proteins from *Oscillatoria* and *Spirulina* species, only 16 to 24% are identical to those in the heterocystous species, but their membership of the same phylogenetic group is established through their higher homology, 31 to 36% identical (52 to 57% similar) to GvpC in *Microcystis* sp. In *Dactylococcopsis salina* there are two additional proteins, one of 17 kDa and the other of 35 kDa; remarkably, the first 24 amino acids in these two proteins are identical to one another, while the subsequent sequences are different (78). Neither of the *Dactyloccopsis* proteins showed significant homology to the other GvpC proteins (Fig. 7).

Both the comparisons, of GvpA and GvpC sequences, indicate a pattern of homology that is consistent with what is known of the phylogeny of these organisms. There is therefore no direct evidence from the sequence data (e.g., of more similar sequences in less closely related organisms) that would support the idea that lateral transfer of *gvp* genes has occurred.

The large variation in GvpC sequences is compatible with an ancient ancestry of this protein. GvpA must, then, have evolved either more recently or more slowly. The original gas vesicles must have had GvpA, because that is what principally forms them, but it is possible that they lacked GvpC. It follows that GvpC cannot be an older constituent of the gas vesicle and that the higher degree of similarity of GvpA in different organisms must be due to a higher level of sequence conservation (78). The small (70-residue) GvpA molecule has to provide not only most of the structure of the gas vesicle but

also most of its properties; this must have imposed many limitations on its evolution. Another limitation has been imposed by duplication of this gene, as discussed below (see Multiple Copies of the *gvpA* Gene). The limitations on evolution of GvpC, which "merely" has to form ties between GvpA, may be fewer. In summary, the limited sequence data suggest that within the bacteria the genes encoding both proteins were inherited linearly rather than acquired by lateral transfer.

By an extension of the same argument, GvpA and GvpC in halophilic archaea may have evolved in the same way: their sequences are more divergent from the cyanobacterial ones, in keeping with their deeper origins in the phylogenetic tree. There are, however, several features of the *gvp* genes in halobacteria that suggest that we should keep open the possibility of acquisition by lateral transfer from a bacterium. First, the homology with cyanobacterial GvpA is high; second, although homology of the N-terminal sequence of *Halobacterium* GvpC is very low (<9% identical), the section containing the 31- to 41-residue repeats may have significant homology with the 33-residue repeats in cyanobacteria (106) (Fig. 10); third, the promoter of the *gvpA* gene is of the bacterial (*Escherichia coli*) type (39); and fourth, the *gvpA*, *gvpC*, and *gvpN* genes are in the same arrangement in the operon, although the *gvpF*, *gvpJ*, *gvpK*, and *gvpL* genes are not (cf. Fig. 4 and 9). In some halobacteria, *gvp* operons are located on plasmids, which might have served as vehicles for lateral transfer between organisms. There is evidence that such transfers have occurred between halobacteria (58).

Information is now needed on the homology of other *gvp* genes in other groups of organisms; Gvp sequences from *Methanosarcina* spp. would be particularly valuable. The phylogenetic relationships of the gas-vacuolate organisms should be established by sequencing their other proteins and rRNAs. Sadly, of the huge body of nucleotide sequence information available, little is from organisms that possess gas vesicles.

**Possible origin from viruses or other structures.** Gas vesicles may have evolved by modification of the protein of some other structure, such as the coats of cylindrical viruses (199, 231). A computer search of data bases has revealed that there is a section of the vesicular stomatitis virus spike glycoprotein G in which the amino acid residues are 29% identical (49% similar) to those in *Anabaena* GvpA (66). There then remains the problem of determining which came first (the virus or the gas vesicle). Other structures that invite morphological comparisons include spinae (53) and various tubular inclusions found in cyanobacteria (101).

## Speculations on Evolution of Gas Vesicle Morphology

It can be argued that the first gas vesicles evolved in halophilic microorganisms (either bacteria, archaea, or their ancestors) that lacked turgor pressure (230, 240) and lived in shallow brine pools, where they encountered little hydrostatic pressure. Under the low pressure in such organisms, the structure formed would have required only a very low critical pressure. From this primitive weak gas vesicle could have evolved a stronger gas vesicle capable of withstanding higher pressure generated by either turgor or depth (see Modifications of GvpA, below). Note that the argument for the origin of gas vesicles in turgorless cells holds irrespective of whether there had subsequently been lateral transfer of the gas vesicle gene between groups of organisms. Had lateral transfer occurred, however, the gas vesicles encoded by the gene would have survived only if they had been strong enough to withstand the pressure encountered in the new recipient organism. Transfers would have been more likely to succeed from cells of high turgor to those of lower turgor than would transfers in the opposite direction.

In passing, it is worth commenting that the same considerations apply to the evolution of the cell wall. A cell wall that developed in a turgorless halophile would have been free of stress; more complex stress-bearing walls would have evolved subsequently in response to natural selection in brackish and then freshwater environments (247).

**Modifications of GvpA.** The first, weak gas vesicles may have had rib-forming proteins (precursors of GvpA) with alternate β-chains of the same length, which consequently crossed the rib at right angles, a common arrangement in silks and other fibrous proteins. A mutation that resulted in alternating β-chains differing in length by one dipeptide (e.g., by the deletion of a dipeptide or the change in the position of a β-turn) would have caused the β-chains to tilt at an angle of 54° to the rib. With the chains thus oriented within 1° of the engineers' magic angle of 55°, the strength of the structure should have been increased. If the location of the β-turns were known, it would be possible to test the idea that the angle does affect strength, by making mutations of the protein that gave alternate chains of equal length. This would not, however, provide evidence that evolution proceeded by this route.

Subsequent changes in GvpA that resulted in wider gas vesicles, allowed by the proposed gain in strength, would have permitted gains in the economy with which buoyancy was provided. Changes that resulted in narrower, stronger gas vesicles would have enabled gas vesicles to provide buoyancy in cells that had higher turgor pressure or in cells that inhabited deeper lakes.

**Multiple copies of the *gvpA* gene.** The amount of GvpA required to provide enough gas vesicles to make a cell buoyant may exceed 5% of the total cell protein (see above). In a cell with 2,000 genes, the average proportion of the total protein produced by each would be 0.05%, and the required transcription rate from a single *gvpA* gene would therefore exceed the average rate by 100 times or more, assuming a uniform rate of turnover. If transcription had been the rate-limiting step in gas vesicle production, there would have been selection for individuals with multiple copies of *gvpA* (234).

From the cyanobacterium *Anabaena flos-aquae* and the prosthecate bacterium *Prosthecomicrobium pneumaticum*, mutants have been isolated that produce fewer gas vesicles than the wild types and insufficient to make the cells buoyant. It was suggested that the wild types might have two or more cistrons of the gas vesicle gene (234, 235). Evidence for multiple copies of *gvpA* has now been provided by gene sequencing in *Calothrix* spp. (37), *Anabaena* spp. (82a), and *Halobacterium* spp. (209).

If a single *gvpA* gene provides insufficient protein to make gas vesicles fast enough to provide positive buoyancy, of what selective value would have been the original single *gvpA* gene? The presence of some gas vesicles would have decreased buoyant density and decreased sedimentation velocity. As discussed above (see Regulation of Gas Vesicle Production), there are examples of extant cyanobacteria with too few gas vesicles for buoyancy, e.g., *Pseudanabaena* sp., which is known to possess only one *gvpA* gene (35). The general question of selection of characters that provide only marginal advantages is discussed by Darwin (38) and Dawkins (42).

There is an idea that after duplication of a gene has occurred, the mutation of the two genes could occur at a higher rate because disadvantageous mutations in one gene would not prove lethal if the other were unchanged. Damerval et al. (37) pointed out that the two copies of the *Calothrix gvpA* gene differ in 18 nucleotides but none of these differences give rise to a change in amino acid sequence. The probability that none

of 18 random mutations will affect the sequence of the translation product is extremely low. Gas vesicle assembly by the wild-type protein might be adversely affected by the presence of a mutated protein (37). Perhaps nonidentical GvpA proteins stack to form a mechanically unstable structure, with the result that amino acid changes specified by only one of two or more *gvpA* genes prove lethal for the gas vesicle. This might explain the low rate of evolution of GvpA in different cyanobacteria. Changes might occur more frequently in mutants having only a single functional GvpA. It is noted that slightly different GvpAs are produced by plasmid and chromosomal genes in halobacteria (209), although it is not known whether these proteins are produced simultaneously in the same cell.

**Modifications of GvpC.** The outer protein, GvpC, may have evolved from one of a number of proteins that, through chance, adhered to sites on GvpA and formed ties between adjacent ribs. The likelihood of this happening is indicated by the finding that lysozyme (which has a short region of amino acid sequence that is similar to part of *Anabaena* GvpC) binds to cyanobacterial gas vesicles (78) and, in doing so, slightly increases their critical pressure (21a). GvpC proteins may therefore be polyphyletic in origin.

Changes in the primitive GvpC that improved its adhesion to GvpA would have been selected if they had increased gas vesicle strength. The increase in the number of 33-residue repeats presumably brought about improvements in the strength or other properties of the gas vesicle. As mentioned above (Function of GvpC: Strengthening the Gas Vesicle), such improvements can be investigated by experiments on gas vesicles reconstituted with recombinant GvpCs containing different numbers of the 33-residue repeats. There are some puzzling aspects to the origin of the repeats.

The 33-residue repeats in GvpC are encoded by 99-nucleotide segments of *gvpC* that themselves comprise partially conserved repeats of 33 nucleotides (37); the 33-nucleotide repeat (encoding 11 residues) might therefore have been the original genetic element from which *gvpC* evolved. The four 33-residue repeats of *Calothrix* GvpC show a homology of 73%, whereas that among the five repeats of *Anabaena flos-aquae* GvpC is even higher, 84%. The homology among the three 11-residue repeats within each 33-residue repeat is much lower (Fig. 5). This indicates that the 33-residue repeats had some conserved property (such as their ability to span a rib) that was not present in the 11-residue repeats.

The homology between the consensus sequences of the 33-residue repeats in *Anabaena* and *Calothrix* spp. is only 52%, considerably lower than the homology between the repeats within each species. This suggests that the sequence of events leading to the extant GvpC in each organism was (i) the presence in the last common ancestor of a gene encoding a protein containing a single 33-residue sequence, (ii) different mutations in the gene giving modified sequences in the ancestral branches of the two organisms, and (iii) replication to give four or five repeats of the modified sequence. What is unconvincing about this scenario is the idea that there should have been no replication of the sequence in the common ancestor and then, independently, replication in both offspring. An alternative is that (i) the gene in the last common ancestor did have repeats and (ii) different mutations occurred in the 33-residue repeats in the two branches leading to *Anabaena* and *Calothrix* spp. but, by some mechanism, particular mutations came to dominate in all of the repeats in each organism. There are other cases in which sequence identity is maintained in tandem arrays of genes, e.g., the genes for rRNA.

Information is now needed on the full sequences of GvpCs in other organisms. In particular, it is important to establish the number of repeats in other organisms. It is noted that there is a correlation between the number of repeats and gas vesicle width in the three organisms (*Anabaena*, *Calothrix*, and *Halobacterium* spp.) for which full sequences are known.

## Natural Selection of the Gas Vesicle

There are two types of questions that can be raised about the natural selection of gas vesicles providing a means of vertical migration in aquatic microorganisms. The first concerns the different forms of gas vesicles that have evolved in different organisms; the second concerns why some organisms evolved gas vesicles, which provide buoyancy, while others evolved flagella, which provide motility. An attempt will be made to supply quantitative answers to these questions.

**Natural selection of gas vesicles of different widths.** Most of the elements of the natural selection of gas vesicle width have been discussed in earlier parts of this review, but they must now be drawn together. The sequence of events in this natural selection is summarized in Fig. 34, which illustrates how the frequency of genotypes that control particular widths may be enriched in a mixed population.

First, consider natural selection for wider gas vesicles that are produced with more economy. Imagine two cells of cyanobacteria in a shallow lake; one cell, *W*, has wide gas vesicles of diameter 84 nm (as in *Anabaena flos-aquae*), and the other, *N*, has narrow ones of diameter 62 nm (as in *Oscillatoria agardhii*). As a consequence of their diameters (equation 10; Fig. 20 and 21), these gas vesicles have critical pressures of 0.6 and 1.0 MPa, respectively. The cells remain in the epilimnion by producing enough gas vesicles for neutral buoyancy, which in *W* requires 5% of the cell protein in the form of Gvp and in *N* requires 7% (see Amount of Gas Vesicles Required for Buoyancy, above). This extra 2% of protein makes no direct contribution to cell growth; suppose the consequence of this is that the cell generation time of *W* is 2% shorter than that of *N*, say 49 h instead of 50 h. After 50 generation times of *W* (i.e., 2,450 h or about 15 weeks, the length of the growing season), *W* could leave a maximum of $2^{50}$ cells while *N* could leave $2^{49}$, half as many. Assuming that cells of both types were subject to the same loss processes (e.g., by predation) this *W*:*N* ratio of 2:1 would be maintained until the next growth season in the following year. (Adams and Carr [2] made a similar analysis of the burden of producing heterocysts.)

Next consider counteracting natural selection for narrower gas vesicles that have greater strength. Imagine the same two cells in the epilimnion of a 70-m-deep lake during the summer period of stratification. After 15 weeks the number of progeny of *W* would again be twice that of *N*. When the lake destratifies in the winter, the stronger gas vesicles in the progeny of *N* will survive circulation to all depths without collapse but the weaker gas vesicles in the progeny of *W* will collapse when they are mixed to below a critical depth, $z_c$ = 20 m, where the combination of hydrostatic pressure ($p_h > 0.2$ MPa) and turgor pressure ($p_t$ = 0.4 MPa) exceeds their critical pressures. When the lake restratifies in the following spring, all of the surviving progeny of *N* could float into the epilimnion to form an inoculum for the next season's growth. Only a proportion of the surviving progeny of *W*, say 25% (depending on such factors as the proportion of water in the lake that is above $z_c$), would remain floating in the epilimnion, while the rest would sink. The ratio of *W*:*N* in the inoculum for the following year would be 1:2, the reverse of that in the shallow lake.

The numbers in this example are imaginary but feasible: the method of quantitative analysis indicates that natural selection

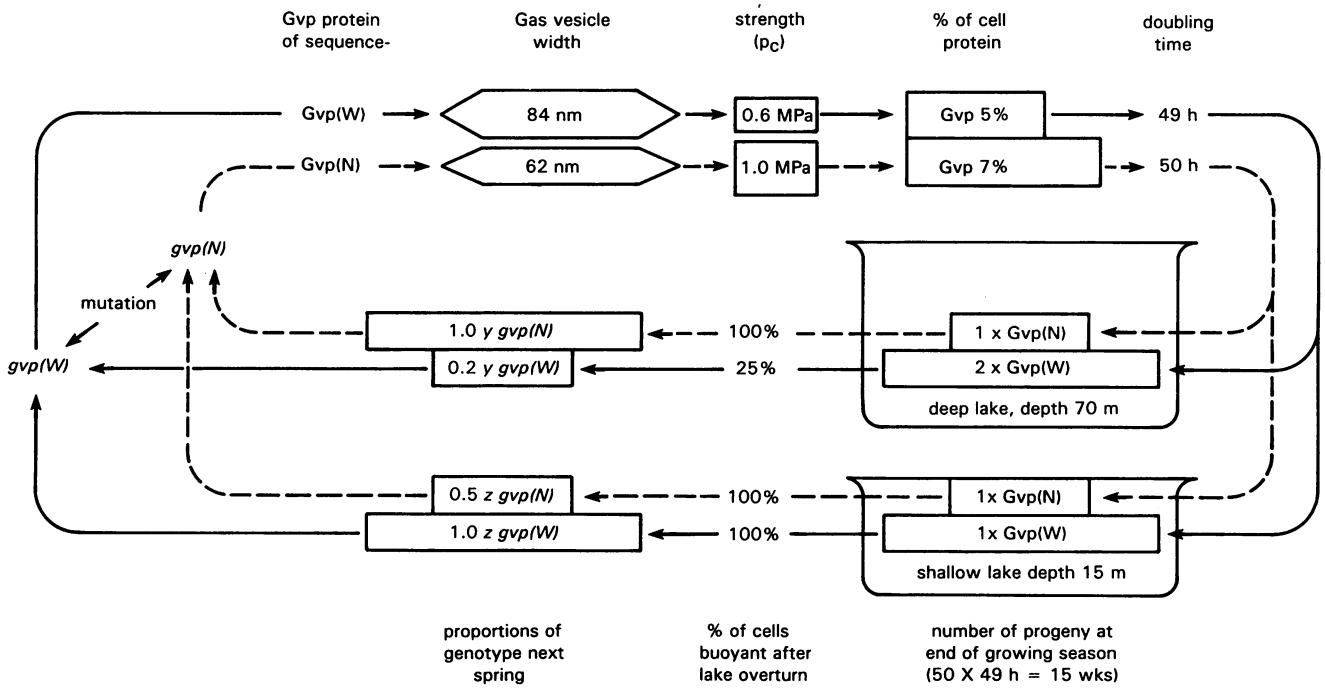


FIG. 34. Natural selection of gas vesicle width determined by the counteracting factors of economy and pressure. To achieve buoyancy, cyanobacterium N, with a mutant form (*N*) of a *gvp* gene that specifies the production of stronger and narrower gas vesicles (like those in *Oscillatoria agardhii*), must devote 2% more of its cell protein to gas vesicle production than does the wild-type W, which has weaker, wider gas vesicles (like those in *Anabaena flos-aquae*). Consequently, the cell generation time of W is 2% shorter, and after 50 generations of growth in a shallow lake it leaves twice the number of progeny to form the inoculum of the next season's population. The same occurs in a deep lake, but after overturn 75% of W, with their weaker gas vesicles, may lose their buoyancy and be lost to the sediment; twice the number of N will then remain in suspension.

for one form or the other could have a profound effect on the ratio of the two types within a few years. These conclusions depend on the validity of a number of assumptions. It has not been demonstrated, for example, how the growth rate of a cyanobacterium is affected by the proportion of its protein that it invests in gas vesicles. It should be possible to determine the effect by comparing the growth rates of mutants with altered gas vesicle content, although in other organisms it has proved difficult to demonstrate the effects of a "protein burden" on growth rate (121, 150). As Mayr has discussed, in the real world each organism in a mixed population has a thousand or more traits in which it may be superior or inferior to the mean of the population; the probability that the organism will survive and reproduce depends on the number of such superior traits (151).

**Gas vesicle versus flagellum.** The buoyancy provided by gas vesicles enables bacteria to rise up toward the water surface, to remain in suspension, or to perform vertical migrations in water. In theory, swimming by means of flagella might provide an alternative means of doing the same. Flagellar movement is more versatile, of course, because it also permits movement in the horizontal plane and tactic or phobic responses to directional stimuli. Why, then, do some microorganisms have gas vesicles rather than flagella? I suggest three possible reasons: (i) that the genetic information for production of flagella does not occur in some groups of procaryotes; (ii) that there are difficulties in sustaining upward migration by flagellar swimming; and (iii) that for some procaryotes, gas vesicles provide a cheaper or faster means of transport.

**(i) Occurrence.** Evolutionary theorists explain the absence of a certain trait in terms of historicity (275), i.e., that the genetic information required for its production either has never existed or has been irretrievably lost from that evolutionary line. In some groups of bacteria flagella are unknown, and the gas vesicle may provide the only available means of vertical movement. Flagella have not been found in cyanobacteria, for example, although Waterbury et al. (269) have discovered a few marine species that are capable of swimming. In other groups, such as the purple and green sulfur bacteria, both gas vesicles and flagella are present, although particular species usually have exclusively either one or the other of these structures (31). *Lamprocystis roseopersicina* and *Lamprobacter* sp. have both, although at different stages of their life cycles (176). The heterotrophic gas-vacuolate bacterium *Ancylobacter* (*Microcyclus*) *aquaticus* was originally described as being nonmotile; some strains were found to produce flagella, however, but usually only under conditions in which gas vesicles were not produced (144). Chemotactic swimming while floating would usually be self-defeating.

Halobacteria possess both gas vesicles and flagella contemporaneously, although they seem to use them for different purposes. As discussed above, the gas vesicles may function in buoying cells to the oxygenated layers of the brine surface; the flagella are used in phototaxis (79). I have observed that the buoyant cells of gas-vacuolate halobacteria accumulate at the surfaces of liquid cultures by floating up, but gas-vacuoleless mutants do not accumulate at the surface by swimming.

**(ii) Sustained upward movement.** The gas vesicle can provide sustained upward transport for days on end and move cells over considerable depths. The prime function of the flagellum in bacteria is executing chemotaxis (48). Usually the concentration gradients of the attractant or repellent chemicals would

have to be quite steep for a cell, swimming at velocities of <0.1 mm $s^{-1}$, to detect a concentration change within a few seconds; the vertical gradients in lakes, extending over many meters, might be undetectable to them, and the cells might therefore lose their way. Most flagellate bacteria have no means of sustaining a vertical swimming direction over long periods. The only exceptions appear to be phototrophs that swim phototactically up a vertical light gradient (170) and magnetotactic bacteria that usually swim downward in seeking the Earth's magnetic pole (12). No magnetotactic forms have been reported from the plankton. Some purple sulfur bacteria that lack gas vesicles use flagellar swimming for vertical migration in lakes where other gas-vacuolate purple sulfur bacteria are simultaneously stratifying (170).

**(iii) Cost.** Perhaps one of the reasons why the flagellum has not replaced the gas vesicle in groups where both may occur is the burden of operating each of the respective structures. I have compared these burdens by the method of cost-benefit analysis developed by Raven for assessing the performance of various organelles in eucaryotic algae (181). There are two costs in operating a flagellum, that of making it and that of rotating it. The common currency in which these costs can be compared is in units of energy expenditure per time (joules per second), i.e., consumption of power (watts).

I start by calculating the energetic cost of making enough gas vesicles to provide neutral buoyancy in a cell of the size of *Escherichia coli*, to avoid subsequent problems of scale. The cell has a wet mass of $9.5 \times 10^{-16}$ kg (156); if the density were 1,060 kg $m^{-3}$ (as in *Anabaena flos-aquae*), the volume would be $8.96 \times 10^{-19}$ $m^3$, and the total ballast mass $M_c - M_w = 5.56 \times 10^{-17}$ kg. The volume of gas, of density 1 kg $m^{-3}$, needed to offset $M_c - M_w$ is $5.57 \times 10^{-20}$ $m^3$; to enclose this in an *Anabaena* gas vesicle containing 130 kg of Gvp per $m^3$ of gas (253) requires $7.24 \times 10^{-18}$ kg of Gvp. The energy cost of making this protein, $E_g$, is calculated by multiplying this mass by 883 kJ per mol of protein C (181) and the carbon content of Gvp (44.4 mol $kg^{-1}$) (85): $E_g = 2.84 \times 10^{-10}$ J. The rate of energy expenditure in gas vesicle synthesis then depends on the generation time ($G$) of the cell: when $G$ = 1 day, the rate is $\ln 2 \times (E_g/86{,}400)$ $s^{-1} = 2.3 \times 10^{-15}$ W; when $G$ = 40 min, the rate is $\ln 2 \times (E_g/2{,}400)$ $s^{-1} = 82 \times 10^{-15}$ W.

The energetic costs of making a flagellum are calculated in a similar way. The volume of the *E. coli* flagellum, a tube of 8 μm in length, 10 nm in diameter, and 3 nm in bore (156), is $2.29 \times 10^{-21}$ $m^3$; multiplying by the density of protein (1,330 kg $m^{-3}$) gives the mass, $3.04 \times 10^{-18}$ kg, requiring an energy input (calculated as above) of $1.19 \times 10^{-10}$ J. The rate of energy expenditure in making a flagellum is therefore $0.96 \times 10^{-15}$ W when $G$ = 1 day and $34 \times 10^{-15}$ W when $G$ = 40 min; these rates are 2.4-fold lower than for the gas vesicle. To be added to these costs, however, are the costs of rotating the flagellum. A single rotation consumes 1,240 protons (155), which at 0.15 eV per proton and $1.6 \times 10^{-19}$ J per eV represents a power consumption of $2.98 \times 10^{-15}$ W. The total cost then becomes $3.9 \times 10^{-15}$ W at $G$ = 1 day, which is now 1.7-fold higher than the cost of gas vesicle production. At $G$ = 40 min, however, the total power requirement for the flagellum is $37 \times 10^{-15}$ W, which is less than half that of the gas vesicle construction costs. At a generation time of $G$ = 10.7 h, the power requirement for gas vesicle production is the same as that for production and operation of the flagellum, $5.13 \times 10^{-15}$ W.

Many other factors could also be included in such an analysis: cells may produce up to twice the number of gas vesicles required for neutral buoyancy (see above) (162, 212, 261); *E. coli* produces on average 3.3 flagella per cell (155), and sometimes as many as 10 (156), whereas other bacteria may produce many more; in energy-starved cells the rate of flagellar rotation falls (155); collapse of gas vesicles and shearing of flagella may introduce additional replacement costs. What transpires from this analysis, however, is that on the basis of energy costs alone for maintaining cells in suspension, there should be natural selection for the static gas vesicle in slowly growing organisms and for the dynamic flagellum in rapidly growing ones. This prediction seems to correspond to the occurrence of these structures, e.g., gas vesicles in slowly growing cyanobacteria and flagella in rapidly dividing heterotrophic bacteria. (The outcome of these calculations is parallel to that found by Raven [181], who, in comparing costs of osmoregulation, found that the static cell wall provided a cheaper solution in slowly dividing cells while the dynamic contractile vacuole was cheaper in rapidly dividing ones.)

I have made similar comparisons with flagellar swimming in eucaryotic algae. Dinophytes, for example, devote a much smaller proportion of their cell mass to flagella than do bacteria (182), and the consequence is that only in cells with a generation time of weeks or even months would the gas vesicle compete in energetic terms. This may be one of the reasons that gas vesicles or their functional analogs are not found in eucaryotic microorganisms.

A final factor that should be mentioned in comparing the flagellum and gas vesicle is the velocity of movement. Cells of *E. coli* swim at up to 20 μm $s^{-1}$. Individual cells of cyanobacteria float or sink at <2 μm $s^{-1}$ (41), and filaments float or sink at <10 μm $s^{-1}$ (249), depending on their size and density, but colonies of such cells may move much faster, at rates exceeding 1 mm $s^{-1}$ (237, 263). These high flotation velocities, which are purely a consequence of size (Stokes's law), are necessary for the daily migration over depths of many meters in stratified lakes. Colonies are formed not only by planktonic cyanobacteria but also by some of the purple bacteria (*Thiopedia* spp.) and methanogenic archaea (*Methanosarcina* spp.).

## CODA

The investigation of gas vesicles has taken the reductionist pathway used in the study of other biological organelles, from discovery in organisms growing in natural ecosystems through exploration by light and electron microscopy, the analysis of chemical and physical structure, and the isolation of genes responsible for production and regulation. What distinguishes the studies on gas vesicles is the way in which this information has been taken back, via physiological studies, to contribute to the understanding of the ecology of the organisms. There has been a incentive to do this because the function of gas vesicles, buoyancy, has little relevance in laboratory cultures but is involved in such important phenomena as stratification, waterbloom formation, and the succession of populations in natural ecosystems.

There are other instances in which topics have started with ecological studies and been pursued to molecular detail but have not made the return journey. An example is the study of chromatic adaptation, which started with Englemann's observations on the coloration of intertidal algae (56), progressed to the discovery that in cyanobacteria the ratio of phycoerythrin and phycocyanin was modified in response to the wavelength of illumination (82), and culminated in the characterization of the many proteins that make up the phycobilisome (73). However, there seems to have been only one demonstration that cyanobacteria perform chromatic adaptation in lakes (67). Likewise, the structure of the bacterial flagellum, the mechanics of motility, and the mechanism of chemotaxis are known in

impressive detail (48), and yet how little is known about the excursions of flagellate bacteria in natural waters. The natural selection of these and many other organelles can be understood only by extending their investigation to the natural habitat. Fortunately, there is now a movement for molecular biologists to apply their trade in natural ecosystems.

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